# APPLICATIONS OF ALPHA-AMYLASE CORN IN THE DRY GRIND PROCESS FOR FUEL ETHANOL PRODUCTION

BY

BRUNO JAVIER GUAGLIANO

THESIS

Submitted in partial fulfilment of the requirements
for the Master of Science in Agricultural and Biological Engineering
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2016

Urbana, Illinois

Master's Committee:

Professor Vijay Singh, Chair
Professor Kent Rausch
Professor Mike Tumbleson

## Abstract

Dry grind processing for fuel ethanol production from corn faces obstacles that limit profitability and sustainability. High temperatures required for liquefaction demand large thermal energy inputs and produce slurries with high final viscosities. These viscosity values increase power consumed by pumping systems and limit slurry solids contents to 32% w/w. Furthermore, enzymes needed for converting starch into glucose account for a considerable fraction of total production costs. To address these issues, inclusion of amylase corn was evaluated. This corn contains alpha-amylase enzyme and therefore obviates the need for addition of exogenous alpha-amylase.

We analyzed the effects of low liquefaction temperatures (65 and 75˚C) on final ethanol concentrations. Corn feedstock included 15% amylase corn and had 32% (w/w) solid loading. Samples with amylase corn (15%) liquefied at 75˚C resulted in ethanol yields similar to those from only yellow dent corn liquefied at 85˚C.

Viscosity profiles were evaluated at 36% (w/w) solids loading during liquefaction. The profiles of mixtures with 15% amylase corn were compared against two treatments of only yellow dent corn, each liquefied with an exogenous commercial alpha-amylase. Initial and peak viscosities of 15% amylase corn samples were higher than those observed in the yellow dent corn treatments. However, final viscosity of alpha-amylase corn treatment was lower than in one of the yellow dent corn treatments and similar to the remaining one.

Corn mixtures with 32 and 36% (w/w) solids loadings and 15% amylase corn were combined with five commercial glucoamylases for evaluating maximum ethanol yields. Ethanol concentrations ranged from 17 to 19% v/v. Ethanol levels obtained in samples with amylase

corn and certain glucoamylases were not different from those observed in samples with only yellow dent corn and conventional enzymes. Due to high residual glucose levels, some treatments with 32% (w/w) solid loading were tested reducing the liquefaction times from 150 to 90 min. Reduced liquefaction temperatures resulted in lower concentrations of residual saccharides.

Inclusion of amylase corn (15%) reduced the liquefaction temperature by 10˚C, resulted in low post liquefaction viscosities and eliminated the need for exogenous alpha-amylases. Addition of alpha-amylase corn in the dry grind process can result in energy savings and may reduce overall processing costs.

## Acknowledgments

I would like to thank my advisor, Dr. Vijay Singh, for giving me the possibility of being part of his research group. His deep knowledge, thoughtful advice and persistent optimism helped me move forward in my research and achieve my academic goals.

I would like to express my gratitude to my thesis committee members, Dr. Kent Rausch and Dr. Mike Tumbleson. Their thorough evaluation and deep insights on academia and life made my stay here an enriching experience.

I am thankful to my colleagues, Dr. Haibo Huang, Dr. Deepak Kumar, Dr. Ming-Hsu Chen, Pavel Somavat, Divya Ramchandran, Sun Min Kim, Zhaoqin Wang and Wei Liu for receiving me into their workgroup. Their kindness as well as work ethic made my experience at UIUC enjoyable and motivating.

I thank Lenore Matthew for her love and constant support at all times, especially in difficult ones. Her intelligence and unbreakable will helped me thrive in this program at a prestigious institution like the University of Illinois at Urbana-Champaign.

I am grateful to my friends in Urbana-Champaign. Our many reunions made me laugh, relax, clear my mind and remember that life does not only consist of work and responsibilities. You helped me stay focused not only from an academic point of view but also from a personal one.

Last but not least, I want to thank my family and friends in Argentina for their love, support and understanding. I could not have achieved any of this without you. Despite the distance, you are always present in my mind and my heart.

## Table of Contents

**Chapter 1. Introduction ..... 1**

**Chapter 2. Literature Review ..... 5**

2.1. Dry Grind Ethanol Production ..... 5

2.2. Starch Converting Enzymes ..... 7

2.2.1. Alpha-Amylase ..... 8

2.2.2. Glucoamylase ..... 9

2.2.3. Pullulanase ..... 11

2.2.4. Granular Starch Hydrolyzing Enzymes (GSHEs) ..... 12

2.3. Corn Slurry Viscosities in Dry Grind Processing ..... 13

2.3.1. Factors that Influence Corn Slurry Viscosity ..... 13

2.3.2. Problems Caused by Increased Viscosities ..... 14

2.3.3. Methods Available for Reducing Slurry Viscosity ..... 14

2.3.3.1. Exogenous Enzymes ..... 15

2.3.3.2. Endogenous Enzymes ..... 15

2.3.3.3. Granular Starch Hydrolyzing Enzymes (GSHE) ..... 16

2.3.3.4. Jet Cooking ..... 16

2.4. High Gravity Fermentation ..... 16

2.4.1. Advantages of High Gravity Fermentation ..... 17

2.4.2. Limitations of High Gravity Fermentation ..... 17

2.4.3. Practices to Address the Limitations of High Gravity Fermentation ..... 18

**Chapter 3. Starch Liquefaction in the Presence of Amylase Corn ..... 21**

3.1. Introduction ..... 21

3.2. Materials and Methods ..... 22

3.2.1. Materials ..... 22

3.2.2. Methods ..... 22

3.2.2.1. Moisture and Starch Content Analyses ..... 22

3.2.2.2. Dry Grind Procedure ..... 23

3.2.2.3. Metabolite Detection ..... 24

3.2.2.4. Ethanol Yields and Conversion Efficiency ..... 24

3.2.2.5. Experimental Design and Data Analysis ..... 27

3.3. Results and Discussion ..... 28
3.4. Conclusions ..... 32

**Chapter 4. Effects of Amylase Corn on Viscosity Profiles during Liquefaction ..... 33**
4.1. Introduction ..... 33
4.2. Materials and Methods ..... 33
4.2.1. Materials ..... 33
4.2.2. Methods ..... 34
4.2.2.1. Moisture and Starch Content Analyses ..... 34
4.2.2.2. Liquefaction and Production of Viscosity Profiles ..... 34
4.2.2.3. Experimental Design and Data Analysis ..... 35
4.3. Results and Discussion ..... 36
4.4. Conclusions ..... 39

**Chapter 5. Performance of Amylase Corn in Combination with Various Glucoamylases .. 41**
5.1. Introduction ..... 41
5.2. Materials and Methods ..... 42
5.2.1. Materials ..... 42
5.2.2. Methods ..... 43
5.2.2.1. Moisture and Starch Content Analyses ..... 43
5.2.2.2. Dry Grind Procedure ..... 43
5.2.2.3. Metabolite Detection ..... 45
5.2.2.4. Experimental Design and Data Analysis ..... 45
5.2.2.5. Additional Experiment: Reduction of Liquefaction Time ..... 46
5.3. Results and Discussion ..... 47
5.3.1 Effect of Reduced Liquefaction Time on Residual Saccharides ..... 48
5.4. Conclusions ..... 51

**References ..... 53**

# Chapter 1. Introduction

The US ethanol industry has grown rapidly since the adoption of the Energy Independence and Security Act (EISA). This mandate, approved by the American Congress in 2005 and expanded in 2007, was intended to mitigate dependence on foreign oil imports and reduce greenhouse gas emissions. The Renewable Fuel Act (RFA) announced in the EISA required increasing volumes of renewable fuels in transportation fuels, concluding with 36 billion gallons in 2022. Currently, the US is the largest fuel ethanol manufacturer, accounting for 58% of the world's total production. Since approval of the RFA to current day, US ethanol production has increased by 286%. In 2015, production capacity reached a plateau at 15.1 billion gal/yr (RFA 2015a). Nonetheless, ethanol will be an important biofuel in the effort to reach RFA's goals by 2022.

Ethanol is produced from a variety of starch based feedstocks such as corn, wheat, barley and sorghum (Solomon et al 2007). In 2014, over 98% of the ethanol produced in the US was made from corn. The industry employs mainly two technologies for the production of ethanol: wet milling and dry grind. Approximately 90% of US ethanol production is based on the dry grind process while the remaining 10% comes from wet milling (RFA 2015a). The wet milling process is more capital and energy intensive. In wet milling plants, capital cost per gallon can reach values two to four times higher in comparison with those of dry grind facilities (Bothast and Schlicher 2005).

In the dry grind process, corn kernels are ground finely to expose starch granules to enzymatic action. Ground corn is mixed with water, forming a slurry. The addition of alpha-

amylase enzyme to the slurry at high temperatures (80 to 110°C) helps to break down starch's crystalline structure. This process, called liquefaction, yields shorter chain saccharides, known as dextrins. During simultaneous saccharification and fermentation (SSF), glucoamylase enzyme is added to reduce dextrins into fermentable sugars such as glucose, maltose and maltotriose. Yeast is added for fermentation of sugars, yielding a mixture of ethanol, water, organic compounds and solids. Distillation and dehydration of this mixture produce fuel ethanol.

The profitability of a dry grind plant depends on the value obtained from its main selling item, fuel ethanol; the main coproduct is dried distillers grains with solubles (DDGS). Fat, protein and fiber are three times more concentrated in DDGS than in the original kernel. Due to the high nutritional value, DDGS are utilized as animal food. In 2013, beef and dairy cattle were responsible for 79% of US domestic DDGS consumption, while nonruminants accounted for an additional 20%. In the same year, US DDGS exports reached 9.7 MMT, 28% of total US production (RFA 2015b). Additional coproducts are distillers corn oil, recovered after distillation, and carbon dioxide ($CO_2$), captured during the fermentation step.

Various issues affect the corn dry grind ethanol production impacting negatively on profitability and sustainability:

- Depolymerization of starch to dextrins requires temperatures of 90°C. The energy input for corn slurry to reach such temperature is 10 to 20% of the fuel value of the ethanol produced (Robertson et al 2006).

- High priced enzymes required for achieving conversion of starch into glucose account for 5% of total production cost (Kwiatkowski et al 2005).

- Slurry viscosity becomes higher with the solids content (Fan et al 2003) increasing energy costs. Additionally, high initial glucose concentrations in fermentation and corresponding high final ethanol concentrations cause osmotic stress on yeasts. These factors restrict solids contents to 32% w/w (Shihadeh et al 2014), limiting plant throughput and ultimately ethanol production capacity.

Sharma et al (2007) conducted dry grind experiments performing liquefaction and SSF with granular starch hydrolyzing enzyme (GSHE). GSHE, which operates at temperatures between 30 and 48°C, is a combination of alpha-amylase and glucoamylase that hydrolyzes crystalline starch into fermentable sugars. Amylase corn, a special corn variety, produces and stores alpha-amylase in its kernels. By replacing a fraction of a plant's conventional feedstock, the addition of liquid alpha-amylase was no longer needed (Singh et al 2006a; Urbanchuk and Kowalski 2009). Fermentation at high solids (high gravity fermentation, or HGF) can decrease production costs, resulting from reduced water and energy inputs, along with lower equipment volumes. To this end, Shihadeh et al (2014) analyzed HGF processing corn slurries at 40% w/w. *In situ* removal of ethanol via vacuum system helped them control ethanol concentration in the fermentation broth. Utilization of GSHE kept peak glucose concentrations at low levels, limiting yeast inhibition. Final ethanol yields were similar to those of slurries at 30% under non vacuum fermentation. Deventier et al (2005) found final ethanol concentration of mashes at 35% w/w to increase with presaccharification and higher glucoamylase doses. Also, they evaluated the suitability of a variety of yeast strains for HGF. Kaur (2010) assessed the effect of nitrogen sources and dosage on HGF's efficiency.

The incentive for this research arises from the necessity to overcome limitations expressed above and ultimately to enhance the performance of the dry grind process. The main goals of this study were to:

- Determine the minimum temperature that allows achievement of an adequate liquefaction for subsequent saccharification and fermentation, working at varying inclusion rates of alpha-amylase corn.

- Analyze liquefaction viscosity profiles of corn slurries at 36% w/w solids in the presence of amylase corn.

- Evaluate ethanol concentrations from fermenting mixtures of yellow dent and alpha-amylase corn treated with various enzyme combinations at 32 and 36% w/w solids loading.

# Chapter 2. Literature Review

## 2.1. Dry Grind Ethanol Production

As the initial step in dry grind plants, corn is ground with hammer mills for exposing starch granules to enzymatic action. Naidu et al (2007) found grinding with 0.5 mm screen resulted in maximum final ethanol concentrations. The addition of water to ground corn produces a slurry with a solids content of 32% w/w. In the following step, called liquefaction, the slurry is heated to 85 to 95°C for 90 min with the objective of breaking down starch's crystalline structure and producing dextrins. For liquefaction to take place, exogenous alpha-amylase is added to the slurry. The pH is adjusted to 5.75±0.05 with sulfuric acid for alpha-amylase optimum performance. As an alternative to the external addition of alpha-amylase, a transgenic corn type that produces this enzyme endogenously can be mixed with conventional corn to produce the slurry (Singh et al 2006b).

After liquefaction, the corn mash is cooled to 32°C before the next step, SSF. Saccharification requires the addition of glucoamylase and fermentation requires an addition of yeast (*Saccharomyces cerevisiae*). The pH is adjusted to 4.50±0.05 to create adequate conditions for glucoamylase and yeast action. During SSF, glucoamylase reduces dextrins into mono and disaccharides, which are fermented simultaneously by yeast. Once inside yeast cells, sugars undergo the glycolytic pathway (also known as EMP or Embden-Meyerhof-Parnas pathway) turning into pyruvate, energy and reduced nicotinamide adenine dinucleotide (NADH + H+). The Crabtree Effect explains that despite the presence of oxygen, yeast takes the anaerobic fermentative route if glucose is present at high levels. If the concentration of oxygen is reduced

and that of glucose is greater than 0.1% (w/v), yeast generates ethanol and carbon dioxide as primary metabolites (Russell 2003).

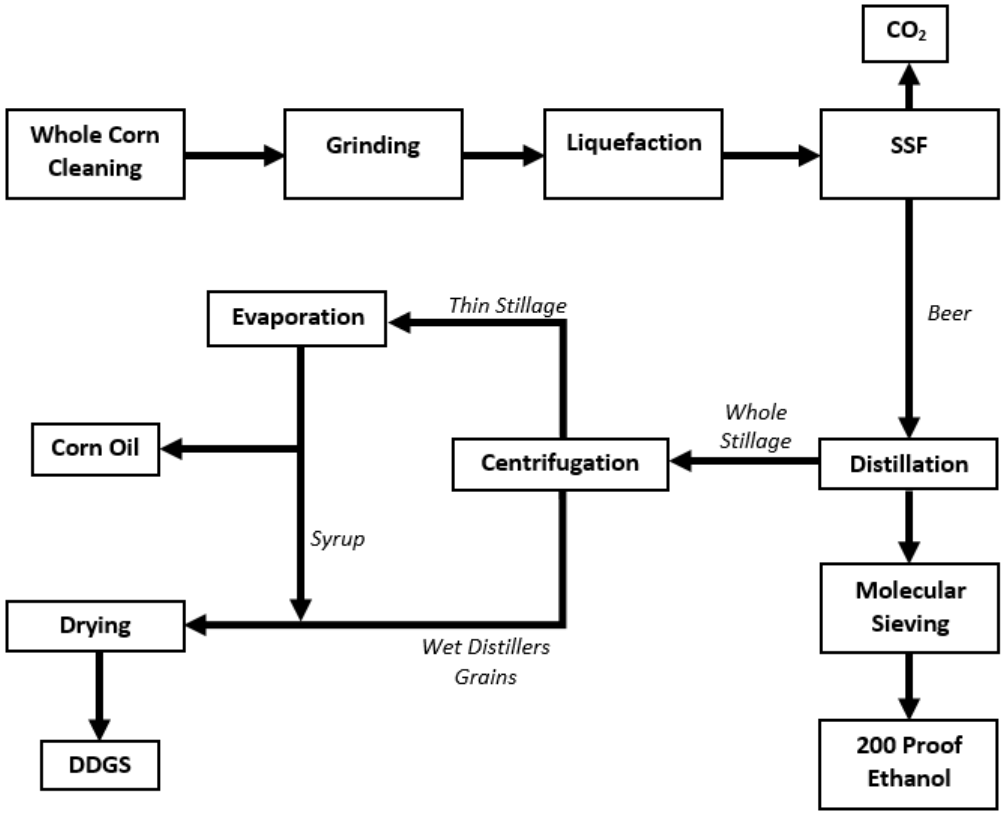


**Figure 2.1.** Dry grind process schematic.

The mixture obtained at the end of the fermentation is called beer, which goes through a distillation step that yields 190 proof (95%) ethanol. The stream is further processed through molecular sieves to remove the remaining water. The resulting 200 proof ethanol is denatured by mixing it with 2% gasoline. This process renders the ethanol undrinkable and thus exempt from beverage alcohol taxes (RFA 2015a). The carbon dioxide released during fermentation may be captured and sold for beverage carbonation and manufacturing of dry ice (Bothast and Schlicher 2005).

The bottom product of the distillation step is called whole stillage. This stream contains water, fiber, oil and protein. Whole stillage is centrifuged to separate liquid from solids obtaining two phases: one phase rich in solids called wet distillers grains (WDG) and other named thin stillage. To save process water and energy, up to 30% of thin stillage is recycled for slurry preparation. The remaining stream is processed through an evaporator system to obtain a syrup containing 30% dry matter. Plants can sell syrup for animal food or combine it with WDG. Nearly 85% of dry grind ethanol plants in the US recover corn oil from thin stillage (RFA 2015a). This coproduct can be utilized as biodiesel production feedstock or as an animal food ingredient. WDG contain 35% dry matter and can be sold to local cattle farms without drying. More frequently, WDG is mixed with syrup and dried to 12% moisture content (US Grains Council 2012). This product is called dried distillers grains with solubles (DDGS). Due to its high fiber content, DDGS utilization as animal food is limited primarily to ruminants. Some methods allow reduction of fiber content and increase protein fraction in DDGS. These features can improve DDGS's market value and bring new opportunities for its commercialization (Singh et al 2004; Srinivasan et al 2005).

## 2.2. Starch Converting Enzymes

*Saccharomyces cerevisiae* (ordinarily known as yeast) is a fungus capable of consuming sugars such as glucose, fructose and maltose (two monosaccharides and a disaccharide, respectively) to produce ethanol via fermentation. However, starch cannot be fermented directly into ethanol by yeast. The reason is the organism's lack of ability to release simple sugars from starch (Power 2003). Originally, starch depolymerization was performed through a process involving the use of acids, high temperatures and pressures. During the 1960's, enzymes

replaced acids for disrupting starch's crystalline structure. The use of these enzymes allowed for a less hazardous and lower energy consuming process. Also, the enzymatic approach yields a higher quality product (Tester et al 2006).

### 2.2.1. Alpha-Amylase

Alpha-amylases, 1,4-α-D-glucan glucanohydrolases, comprise a family of starch degrading enzymes. In the dry grind process, the liquefaction step requires this enzyme to be active at high temperatures (80 to 110°C). Weemaes et al (1995) tested three microbial sources of alpha-amylase concluding that *Bacillus licheniformis*, a mesophilic bacteria, produced the enzyme with the highest thermostability. For this reason, alpha-amylase for starch liquefaction is manufactured primarily from this type of microorganism.

Alpha-amylases perform optimally at temperatures and pH values ranging from 85 to 95°C and 5 to 6, respectively. This enzyme is endo acting, which means it attacks the inner region of amylose and amylopectin chains. It splits α-1-4 glycosidic bonds randomly yielding water soluble saccharides of varied length (van der Maarel et al 2002). The action pattern of this enzyme is limited to α-1-4 bonds; it cannot cleave α-1-6 glycosidic bonds and skips the branching points in amylopectin. Due to this limitation, action of alpha-amylase on amylopectin results in branched products (Power 2003).

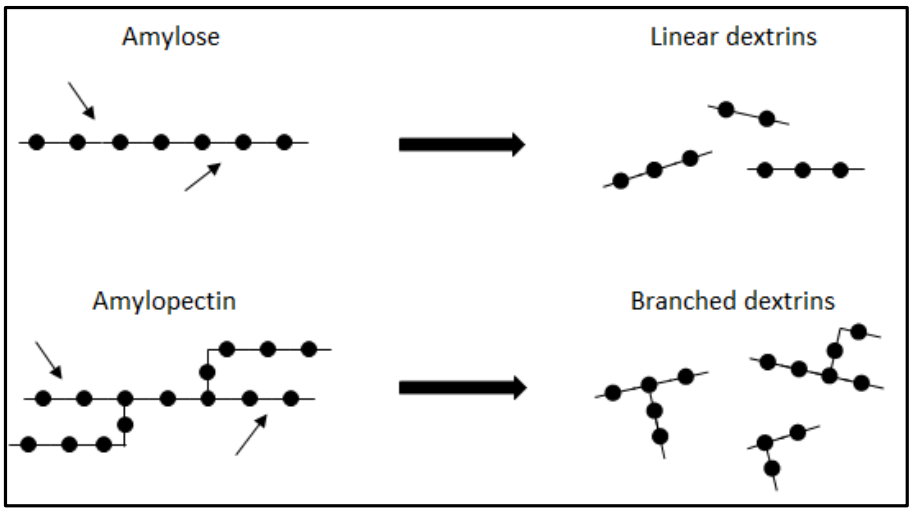


**Figure 2.2.** Action pattern of α-amylase. Smaller arrows indicate α-1-4 bonds attacked by the enzyme.

### 2.2.2. Glucoamylase

Glucoamylases, amyloglucosidases or 1-4-α-D- glucanohydrolases, are employed in the ethanol industry for saccharification of liquefied starches. This process yields simple sugars that are fermentable by yeast.

Although glucoamylases can be obtained from many fungal sources, they usually are produced from *Aspergillus niger* and *Rhizopus species* (Nigam and Singh 1995). Fungal enzymes are less thermotolerant than those produced from bacterial sources (Power 2003). Glucoamylase from *Aspergillus niger* has an optimum pH of 4.2 and is stable at 60°C (Crabb and Mitchinson 1997).

Technological advances allow the production of genetically engineered yeast strains that produce glucoamylase and grow by utilizing soluble starch as its only carbon source (Kondo et al 2002). This type of yeast can convert raw starch into ethanol, eliminating or reducing the addition of exogenous glucoamylases (Marin-Navarro and Polaina 2011).

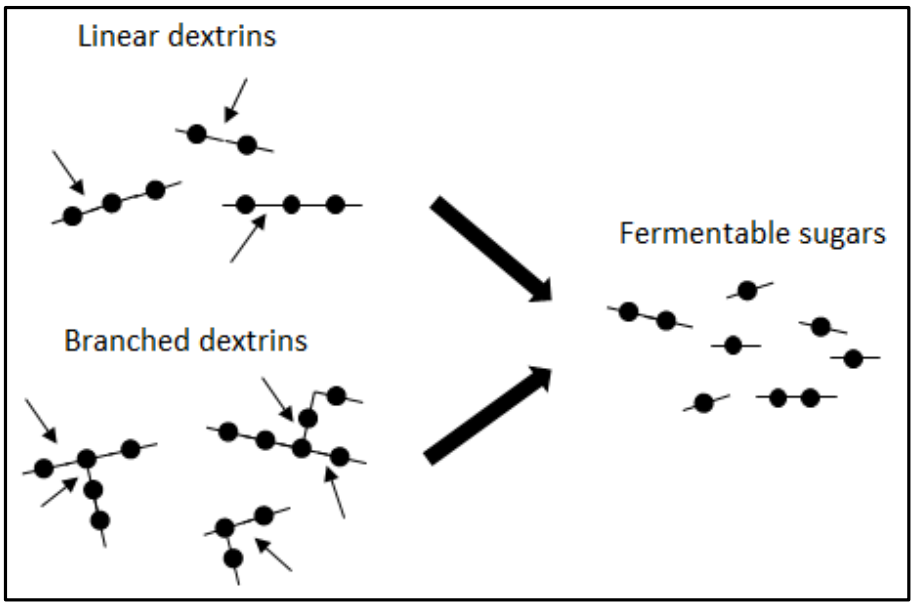


**Figure 2.3.** Action pattern of glucoamylase. Smaller arrows indicate α-1-4 and α-1-6 bonds attacked by the enzyme.

Glucoamylase functions as an exo acting agent attacking both α-1-4 bonds as well as the α-1-6 bonds present at the branching points of oligosaccharides (Crabb and Mitchinson 1997). In contrast with alpha-amylase (an endo acting enzyme), glucoamylase splits bonds in a sequential rather than in a random fashion. It cleaves glucose monomers successively from the nonreducing end of the saccharide, making them available for fermentation via yeast (Saha and Zeikus 1989). Despite the capability of this enzyme to hydrolyze both α-1-6 and α-1-4 bonds, the former are attacked at slower rates than the latter (Saha and Zeikus 1989; Power 2003; Norouzian et al 2006). Power (2003) and Hii et al (2012) suggest these rates are 20 to 30 and 50 times lower, respectively. Furthermore, the hydrolytic power of glucoamylase is affected by substrate molecular structure, molecular size and by the next bond in sequence. Glucoamylase has higher affinity for longer chains (Saha and Zeikus 1989).

### 2.2.3. Pullulanase

Pullulanases, pullulan-6-glucanohydrolases, are a group of debranching enzymes occasionally combined with glucoamylase for converting starch mashes into glucose. Pullulanase is an endo acting enzyme capable of cleaving α-1-6 glycosidic linkages of pullulan and branched polysaccharides at random. This process results in a mixture of linear dextrins, maltotriose being the primary product (Hii et al 2012).

Glucoamylases hydrolyze α-1-6 bonds at slower rates than α-1-4 bonds. Also, they catalyze glucose reversion reactions that yield compounds such as isomaltose, isomaltotriose and other α-1-6 oligosaccharides. These products accumulate at the expense of glucose, leading to a decrease of glucose yields (Zhao et al 2015). In a saccharification process that combines pullulanase and glucoamylase, the former hydrolyzes primarily branching points while the latter splits linear chains. For this reason, saccharification may require reduced levels of glucoamylase thus generating fewer reversion products (Norman 1982). Further benefits of pullulanase include the possibility of conducting saccharification at solids contents of 40% w/w (Stominska and Maczynski 1985; Hii et al 2012).

Pullulanases from microbial sources (eg, from *Bacillus* species) are preferred due to their high specificity towards the hydrolysis of α-1-6 bonds (Hii et al 2012). Optimum temperature for this enzyme is 60°C and ideal pH is 4.5 to 5.5 (Norman 1982). These optimum operational conditions match those of glucoamylase from *Aspergillus niger*, making these enzymes suitable for simultaneous performance.

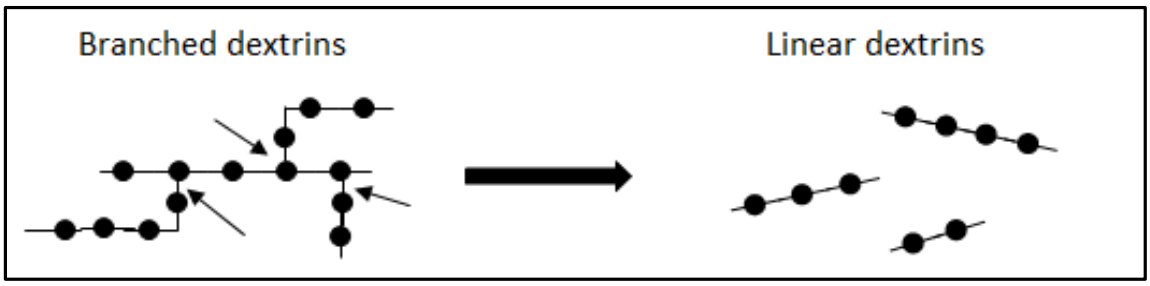


**Figure 2.4.** Action pattern of pullulanase. Smaller arrows indicate α-1-6 bonds attacked by the enzyme.

### 2.2.4. Granular Starch Hydrolyzing Enzymes (GSHEs)

A collection of special alpha-amylases, glucoamylases, alpha-glucosidases and isoamylases are considered granular starch hydrolyzing enzymes (GSHEs) (Robertson et al 2006). GSHEs are capable of hydrolyzing raw granular starches at sub gelatinization temperatures. Depolymerization of starch using GSHEs entails numerous benefits: performing hydrolysis at reduced temperatures can reduce process energy consumption (Robertson et al 2006; Wang et al 2007). Kaur et al (2011) and Wang et al (2007) showed that during SSF, systems treated with GSHEs developed lower glucose concentration peaks than conventional processes. This scenario is beneficial for fermentation, as yeast is subjected to lower osmotic stress and less substrate is available for competing organisms.

Wang et al (2007) tested GSHEs by conducting hydrolysis at 48°C. Subsequent fermentation resulted in ethanol concentrations comparable to those of samples liquefied at 90°C with conventional alpha-amylase. Uthumporn et al (2012) conducted liquefaction with GSHE at 35°C obtaining corn slurries with lower viscosities in comparison to those of heat treated ones.

## 2.3. Corn Slurry Viscosities in Dry Grind Processing

In the dry grind process for ethanol production, corn slurry viscosities before and after liquefaction are critical factors to consider. These viscosities can influence the performance of liquefaction and subsequent steps of the process.

### 2.3.1. Factors that Influence Corn Slurry Viscosity

Various factors influence the viscosity profiles of corn slurries during liquefaction. One is the gelatinization properties of starch, which is dependent on starch granule's rigidity. This rigidity can affect the swelling potential of the granules and the amounts of amylose released into the solution (Sandhu and Singh, 2007).

Liquefaction generates a mixture of amylose and amylopectin hydrolysis products, with sizes ranging from oligosaccharides to large polymeric molecules. In a polymer solution, viscosity depends on interactions between solvent and polymer as well as on properties of the latter, such as structure and molecular weight (Dokic et al 2004). Large concentrations of high molecular size saccharides resulted in high slurry viscosities (Dokic et al 2004; Wang and Wang 2000).

Amylose goes through a process called retrogradation when starch suspensions, such as corn slurries, are heated and cooled. Retrogradation causes the fluid to turn into a gel, due to the development of a permanent elastic network formed by amylose chains. The network's growth rate and length increase with molecular size. Amylose chains comprised of more than 110 glucose monomers tend to retrograde upon cooling of the suspension (Morris 1990). Sandhu and Singh (2007) showed the viscosity of cooked starch suspensions increased after cooling and suggested that aggregation of amylose molecules was responsible for this increase.

On thermally treated wheat starch suspensions, Bagley and Christianson (1982) showed a rapid viscosity increase when the solids content exceeded 16%. This viscosity increase occurred at gradually lower concentrations as cooking temperature became higher.

### 2.3.2. Problems Caused by Increased Viscosities

In high gravity corn mashes, the high concentration of solids resulted in viscous slurries, consequently increasing the energy consumption during liquefaction and fermentation (Thomas et al 1996). Viscous friction is the main source of pressure decrease and pumping power requirements (Sahin 2002). Using Fanning's equation, Bakshi and Smith (1984) explained that as a fluid's viscosity increases, friction losses become higher and more power is required to move it. Additionally, viscosity is a property that affects heat transfer (Sahin 2002).

Dahod (1993) measured the concentration of dissolved $CO_2$ in two fermentation broths of different viscosity. The less viscous broth had a viscosity of 100 cP while the more viscous was in a range of 2000 to 3000 cP. The high viscosity broth retained 25% more $CO_2$ in solution than the less viscous one. High concentrations of $CO_2$ in the fermentation broth can inhibit the performance of *Saccharomyces cerevisiae*. Jones and Greenfield (1982) observed that at $CO_2$ levels over 0.2 atm, yeast cell growth and metabolism were affected. They argued that yeast cell growth inhibition intensified in the presence of ethanol due to a synergistic effect between this alcohol and $CO_2$.

### 2.3.3. Methods Available for Reducing Slurry Viscosity

The primary purpose of the liquefaction step is to hydrolyze partially the starch suspension, reduce its viscosity and turn it resistant to retrogradation (Aiyer 2005). Dokic et al

(2004) stated the saccharide distribution became narrower as dextrose equivalent (a measure of the degree of hydrolysis of a saccharide solution) increased. This resulted in lower solution viscosities. Currently, the industry employs diverse technologies for hydrolyzing starch, therefore, reducing slurry viscosity.

#### 2.3.3.1. Exogenous Enzymes

The simple or combined addition of enzymes from microbial origin aids in the process of starch degradation. These enzymes have varied action patterns, some of them acting sequentially and some randomly on glycosidic bonds. They also differ in the type of bonds they are capable of splitting; some can attack only α-1-4 bonds, some only α-1-6 bonds; others can cleave both kinds. These enzymes act as effective thinning agents, reducing the viscosity of the treated slurry (Aiyer 2005).

#### 2.3.3.2. Endogenous Enzymes

An alternative to the external addition of enzymes is the use of a corn variety that produces and accumulates alpha-amylase within the kernel (Urbanchuk and Kowalski 2009). This kind of enzyme, known as “endogenous alpha-amylase”, is activated in water solution at temperatures above 70°C. Singh et al (2006a) showed that even at inclusion levels of 3%, this type of corn provided sufficient alpha-amylase to obtain lower slurry viscosities than with addition of exogenous enzymes.

#### 2.3.3.3. Granular Starch Hydrolyzing Enzymes (GSHE)

GSHE's are capable of hydrolyzing starch into dextrins at temperatures below 48°C and also can convert dextrins into sugars during SSF (Wang et al 2007). With wheat starch suspensions, Bagley and Christianson (1982) demonstrated that viscosities increased rapidly with cooking temperatures of 60, 65, 70 and 75°C. Based on these concepts, Uthumporn et al (2012) conducted starch hydrolysis at sub gelatinization temperature (35°C) using GSHE's. He observed lower viscosities than for samples treated by conventional hydrolysis.

#### 2.3.3.4. Jet Cooking

The amylopectin present in starch is a largely branched polysaccharide of high molecular weight. This component of starch proved to be sensitive to shearing forces (Dintzis and Bagley 1995). In a particular version of the liquefaction process, enzymes were added and the slurry was pumped through a jet cooker, which rapidly increased the temperature of the mixture to over 100°C by injection of live steam. Extreme shearing forces exerted over the starch granules generated mechanical thinning of the slurry, in addition to the thinning produced by enzymatic action (Aiyer 2005).

Dintzis and Bagley (1995) subjected dent corn samples to hydrolysis treatments of varying nature. Increasing process severity resulted in stronger reductions of slurry viscosity. Jet cooking was the most efficient among the treatments tested.

## 2.4. High Gravity Fermentation

The application of HGF in the ethanol industry is an alternative that can offer multiple benefits, such as the reduction of production costs and utilization of resources. However, the

fermentation of mashes at high sugar concentrations by *Saccharomyces cerevisiae* faces some obstacles that prevent its vast industrial application. Recently, researchers investigated a variety of methods to overcome these limitations and broaden the applicability of HGF.

### 2.4.1. Advantages of High Gravity Fermentation

The fermentation of high gravity mashes offers the possibility of reducing operational and capital costs in dry grind facilities (Shihadeh 2014; Huang et al 2015). The reduction of operational costs is associated with a decrease in the consumption of resources throughout the process. One of these resources is water, as increasing the slurry's solid fraction results in corresponding lower water inputs. Working at 35% solids, evaporation and drying became less energy demanding due to reduced loads on dewatering equipment (Taylor et al 2000). Also, residual disposal cost may be diminished because of lower amounts of water used (Mohagheghi 1992). For a constant ethanol output, HGF can decrease the load in dry grind plants. Reduced loads allow for the utilization of less voluminous processing equipment. Using process simulation, Taylor et al (2010) showed that HGF resulted in capital cost reductions.

### 2.4.2. Limitations of High Gravity Fermentation

High concentrations of solids in dry grind fermentation may lead to problems related to substrate and product inhibition of yeasts. Increasing the sugar concentration in fermentation broth exerts osmotic stress on *Saccharomyces cerevisiae*, resulting in a lower degree of cell multiplication and reduced sugar consumption (Thomas et al 1993). Thatipamala et al (1992) observed the onset of yeast inhibition caused by the substrate to become noticeable at a glucose concentration of 15% w/v. Casey and Ingledew (1986) reported total growth inhibition at 40%

(w/v) glucose concentration. At ethanol levels from 10 to 13% v/v, yeasts began to encounter an inhibitory process (Casey and Ingledew 1986). As ethanol concentrations increased, Brown et al (1981) observed a decline in fermentation rates, as well as in yeast cell growth rate and viability. Despite the presence of sugars, high ethanol concentrations brought fermentation to a complete stop (Mohagheghi et al 1992).

High viscosities hinder the release of the $CO_2$ produced during fermentation, causing greater volumes of this gas to remain in the reactor (Dahod 1993). At dissolved $CO_2$ concentrations above 0.2 atm, yeast cell growth and general metabolic activity were affected negatively (Jones and Greenfield 1982).

In HGF, the lack of yeast nutrients can be detrimental to many aspects of fermentation, including yeast cell growth (Jones and Ingledew 1994b). The stress imposed over *Saccharomyces cerevisiae* may lead to incomplete consumption of sugars (Mohagheghi et al 1992), reduced ethanol yields and low fermentation efficiencies (Huang et al 2015).

### 2.4.3. Practices to Address the Limitations of High Gravity Fermentation

To address yeast inhibition due to high glucose levels, Shihadeh et al (2014) employed GSHE for processing samples at 30, 40 and 45% w/w solids. They conducted simultaneous liquefaction, saccharification and fermentation at 32°C. The highest peak glucose concentration observed was 5.6% w/v, which was lower than the minimum inhibitory level of 15% w/w suggested by Thatimapala et al (1992).

Kaur et al (2011) evaluated post liquefaction viscosities of samples at 35% w/w solids through a modified process. In this process, a combination of alpha-amylase and phytase was

utilized at 55°C for liquefaction, resulting in a viscosity reduction of 80% between conventional and modified methods (2700 and 550 cP, respectively).

Shihadeh et al (2014) developed a vacuum stripping system that allowed removing ethanol from the fermentation media during the reaction. By applying cycled vacuum steps, the fermentation of corn mashes at 30, 40 and 45% w/w solids did not exceed ethanol concentrations of 9.2% v/v, thus avoiding yeast inhibition.

Taylor et al (2010) proposed a system in which the fermentation mash was pumped through a gas stripping column. In this column, a noncondensable gas extracted ethanol from the mash, maintaining its concentration at under inhibitory levels. This gas can be carbon dioxide produced from fermentation, nitrogen or air. Daugulis et al (1991) modeled a liquid extraction process that employed an ethanol selective and water insoluble solvent. Solvent was added directly to the reactor for separating ethanol from the broth, keeping it at low concentrations and preventing yeast inhibition. The ethanol rich solvent coming out of the fermenter was flash vaporized for recovering the alcohol; the solvent was recycled into the fermentation tank.

Detrimental effects on yeast metabolism caused by low availability of free amino nitrogen (FAN) during HGF can be overcome by the inclusion of certain additives. Jones and Ingledew (1994a) showed that different sources of nitrogen, such as ammonium ion and urea, among others, improved the fermentation rate by increasing FAN concentration. Urea was the most economically feasible FAN source for fuel ethanol production. Jones and Ingledew (1994b) observed a stimulation of fermentation rates when adding proteases to HGF mash. It was suggested this effect was caused by FAN released due to the hydrolytic effect of proteases on soluble proteins.

Thomas et al (1994) evaluated the capability of glycine to improve the metabolism of yeast under HGF conditions. Despite being a poor source of nitrogen, glycine allowed yeast to maintain a high cell viability level (above 80%) during fermentation.

# Chapter 3. Starch Liquefaction in the Presence of Amylase Corn

## 3.1. Introduction

In dry grind processing for ethanol production from corn, the first step after cleaning and grinding is liquefaction. The objective of this procedure is to hydrolyze kernel starch and reduce polysaccharide chain length released into the solution. Typically, a conventional liquefaction requires external addition of alpha-amylases at operational temperatures of 85 to 95°C. Energy consumed for depolymerization of starch into short fermentable saccharides equates to 10 to 20% of the energy contained in fuel ethanol (Robertson et al 2006). In a model of the dry grind process, the cost of enzymes accounted for nearly 5% of ethanol's total production cost (Kwiatkowsky et al 2005). If corn cost, the primary expense in dry grind process, is not considered, the contribution of enzymes to production cost increases to 20%.

A transgenic corn, amylase corn, has the capability of producing and accumulating alpha-amylase in the seed endosperm (Urbanchuk and Kowalski 2009). Singh et al (2006a) liquefied corn starch into dextrins by adding 3% amylase corn when operating at 90°C. Addition of this corn at higher inclusion rates could provide enough alpha-amylase for conducting liquefaction at lower temperatures than in the conventional process. Successful liquefaction of starch at low temperatures using amylase corn may translate into cost reductions, due to reduced consumption of energy and exogenous enzymes.

We tested the liquefaction of samples with 15 and 20% amylase corn at temperatures of 65 and 75˚C. The primary objective was determining the minimum liquefaction temperature for which final ethanol concentrations after SSF are not different in comparison with those achieved by the conventional dry grind process.

## 3.2. Materials and Methods

### 3.2.1. Materials

Two corn varieties were provided by a commercial seed company. They were yellow dent and alpha-amylase corn, both harvested in October, 2014. An exogenous alpha-amylase was used for liquefaction in the control treatment. This enzyme had optimum pH and temperature at 5.8 and 85˚C, respectively. Glucoamylase acted as a saccharification agent in SSF. Optimum pH and temperature application ranges recommended by the manufacturer were 4 to 5.5 and 28 to 35˚C, respectively.

Fermentis-Lessaffre Yeast Corporation (Milwaukee, WI) provided active dry yeast (Ethanol Red) for corn slurry fermentation. Sulfuric acid 10*N* used for pH adjustment and urea added as a nitrogen source for yeast nutrition were supplied by Ricca Chemical Company (Arlington, TX) and Fisher Scientific (Fair Lawn, NJ), respectively. Deionized water was produced in our laboratory.

### 3.2.2. Methods

#### 3.2.2.1. Moisture and Starch Content Analyses

Ground corn moisture content was determined through a conventional oven method (AACC International 2000a). A total starch assay procedure (AACC International 2000b) was followed for calculating the starch content of the samples, using an assay kit manufactured by Megazyme International Ireland Limited (Bray, Wicklow, Ireland).

### 3.2.2.2. Dry Grind Procedure

For removing broken kernels and foreign material, corn samples were hand cleaned and sieved over a 12/64" (4.8 mm) round hole sieve. Clean corn was ground in a laboratory hammer mill (Retsch SK100, Glen Mills, Clifton, NJ) operated at 3,420 rpm using a 0.5 mm screen. Samples were stored in plastic bags at 4°C. Determinations of moisture and total starch contents were performed as described in section 3.2.2.1.

To achieve 32% w/w solids content, ground corn samples weighing 100 g (db) were mixed with deionized water at ambient temperature. The pH was adjusted to 5.75±0.05 (optimum pH for alpha-amylase) with sulfuric acid 10*N*. Alpha-amylase was added only to the control treatment, as it contained no amylase corn. This enzyme was applied at the upper level of the manufacturer's recommended dose (0.024 mg/g (db) corn). Liquefaction was conducted in 500 ml stainless steel reactors with continuous agitation in a programmable incubator (Labomat BFA-12, Werner Mathis AG, Switzerland). Samples were held at the selected liquefaction temperature (85, 75 or 65°C) for 90 min and cooled to 32°C before SSF.

After liquefaction, pH was adjusted to 4.50±0.05. Urea was dosed at 2.25 mg urea/g (db) corn. Glucoamylase was added at the upper level of the manufacturer's recommended dose (0.08 mg/g (db) corn), followed by yeast inoculate at 3.3 mg dry active yeast/g (db) corn. SSF was conducted at 32°C in an automatic incubator (New Brunswick Innova 42R Inc/Ref Shaker, Eppendorf, CT) with continuous agitation for 72 hr.

### 3.2.2.3. Metabolite Detection

During SSF, 2 ml samples were collected at 0, 4, 8, 24, 48 and 72 hr and centrifuged (Centrifuge 5415D1, Eppendorf, Germany) at 15,000 *xg* for 5 min. Supernatant liquid was cleared of solid particles by passing it through a 0.2 µm syringe filter (Pall Gelman, Milford, MA) into 1 ml vials. Metabolite analysis was performed through high performance liquid chromatography (HPLC). Filtered liquid was injected into an ion exclusion column (Aminex HPX-87H, Bio-Rad, Hercules, CA) maintained at 50°C. Alcohols (ethanol, methanol and glycerol), sugars (DP4+, DP3, DP2, fructose and glucose) and organic acids (lactic, succinic and acetic) were quantified by a refractive index detector at 35°C (model 2414, Waters Corporation, Milford, MA).

### 3.2.2.4. Ethanol Yields and Conversion Efficiency

A set of equations was developed to estimate ethanol volumes produced during fermentation and total volume of the mash's liquid phase. Combining these expressions with others presented here allowed calculating ethanol yields and fermentation efficiencies. Mean starch concentration (on dry basis) for determining the maximum theoretical ethanol yield was 71.9%. Mean moisture content was 10.2%.

### Theoretical Ethanol Yield

The theoretical maximum volume of ethanol produced by fermentation was calculated as:

$$V_{th} = \frac{w_C\ (wb) \times (1 - M_c) \times X_s(db) \times 1.11 \times 0.51}{\rho_{e\,20^\circ C}} \qquad (1)$$

where $V_{th}$ = theoretical maximum volume of ethanol [L]; $w_c$ (wb) = corn sample weight, on wet basis [kg]; $M_c$ = moisture content; $X_s$ (db) = starch content (db); 1.11 = starch to glucose conversion ratio [kg of glucose/kg of starch]; 0.51 = glucose to ethanol conversion ratio [kg of ethanol/kg of glucose]; $\rho_{e20^\circ C}$ = density of ethanol at 20˚C = 0.789 kg/L.

Theoretical yield (on dry basis) of ethanol was expressed as:

$$Y_{th} = \frac{V_{th}}{w_C \text{ (wb) x } (1 - M_c)} \quad (2)$$

where $Y_{th}$ = theoretical yield of ethanol [L/kg].

**Actual Ethanol Yield**

The fractional concentration of ethanol in the liquid phase was expressed as:

$$C = \frac{V_a}{V_L} \quad (3)$$

where C = fractional concentration of ethanol in the liquid phase; $V_a$ = actual volume of ethanol in the liquid phase [L]; $V_L$ = total volume of the liquid phase [L].

Glycerol produced during fermentation was minimal and considered as a negligible factor on the sample's final volume (Shihadeh et al 2014). For this reason, the liquid phase total volume was calculated as the sum of the volumes of water, corn moisture and ethanol produced.

Another factor considered in developing an expression for the total volume of the liquid phase was the volume decrease that occurred when ethanol and water form a solution (Thomas et al 1996).

Liquid phase total volume was calculated as:

$$V_L = \frac{V_w \text{ x } \rho_{w_{20^\circ C}} + w_C\,(wb) \text{ x } M_c + V_a \text{ x } \rho_{e_{35^\circ C}}}{\rho_{[w/e]_{20^\circ C}}} \quad (4)$$

where $V_w$ = volume of water added [L]; $\rho_{w20^\circ C}$ = density of water at 20˚C = 0.998 kg/L; $\rho_{e35^\circ C}$ = density of ethanol at 35˚C (HPLC's detector temperature) = 0.776 kg/L; $\rho_{[w/e]20^\circ C}$ = density of the water/ethanol mixture at 20˚C [kg/L].

Replacing (4) into (3) and solving for $V_a$:

$$V_a = \frac{w_C\,(wb) \text{ x } M_c + V_w \text{ x } \rho_{w_{20^\circ C}}}{\frac{\rho_{[w/e]_{20^\circ C}}}{C} - \rho_{e_{35^\circ C}}} \quad (5)$$

We can express actual ethanol yield (on dry basis) as:

$$Y_a = \frac{V_a}{w_C(wb) \text{ x } (1\text{-}M_c)} \quad (6)$$

where $Y_a$ = actual ethanol yield [L/kg].

**Conversion Efficiency**

Fermentation efficiency was determined as:

$$F_{eff} = \frac{Y_a}{Y_{th}} \quad (7)$$

### 3.2.2.5. Experimental Design and Data Analysis

Two corn mixtures with different percentages of alpha-amylase and yellow dent corn (15/85 and 20/80%, respectively) were liquefied at 75 and 65˚C. These samples were compared and contrasted with a control treatment of 100% yellow dent corn, liquefied at 85˚C (Table 3.1). All samples were processed under the conditions described in section 3.2.2.2.

**Table 3.1.** Treatments used to determine the effects of low temperature liquefaction of corn mixtures at high inclusion levels of amylase corn in dry grind process.

| Corn Composition [%amylase / %yellow dent] | Liquefaction Temperature [˚C] |
|---|---|
| 20/80 | 75 |
| 15/85 | 75 |
| 20/80 | 65 |
| 15/85 | 65 |
| 0/100 (control) | 85 |

Each treatment was evaluated with three replications. Liquefaction and fermentation conditions, such as pH, temperature and doses of urea, glucoamylase and yeast, were constant for each treatment. Samples were analyzed in duplicate using HPLC. Sugar, alcohol and organic

acid profiles were evaluated to determine effects of low temperature liquefaction and high inclusion levels of amylase corn on final ethanol concentrations and conversion efficiencies. Final ethanol concentrations, residual glucose, ethanol yields and conversion efficiencies were evaluated statistically using SAS (SAS Institute, Cary, North Carolina). Analysis of variance (ANOVA) and Fischer's least significant difference (LSD) tests with significance level of $p<0.05$ were used to compare means among treatments.

## 3.3. Results and Discussion

All treatments had similar fermentation rates during the initial stages of SSF (0 to 8 hr). After 24 hr of fermentation, samples liquefied at 65˚C had lower fermentation rates in comparison with those liquefied at 75 and 85˚C (Fig. 3.1). Sandhu and Singh (2007) observed onset gelatinization temperatures from 66 to 69˚C for starch from different corn varieties. Therefore, the liquefaction of samples at 65˚C may have resulted in partial gelatinization and thus incomplete starch hydrolysis. This phenomenon can limit the amounts of fermentable sugars available for yeasts and have a detrimental effect on final ethanol concentrations.

**Table 3.2.** Final ethanol and glucose concentrations (means of three observations).

| Treatment | | Parameters | |
|---|---|---|---|
| Corn Composition [% amylase / % yellow dent] | Liquefaction Temperature [˚C] | Final EtOH Conc.[a,b] [%v/v] | Final Glucose Conc. [%w/v] |
| 20/80 | 75 | 19.1 a | 0.0 |
| 15/85 | 75 | 19.0 a | 0.1 |
| 20/80 | 65 | 15.9 b | 0.0 |
| 15/85 | 65 | 16.0 b | 0.0 |
| 0/100 (control) | 85 | 18.7 a | 0.2 |

[a] Means followed by the same letter in the same column are not different ($p<0.05$).

[b] LSD for final ethanol concentration was 0.8 % w/v.

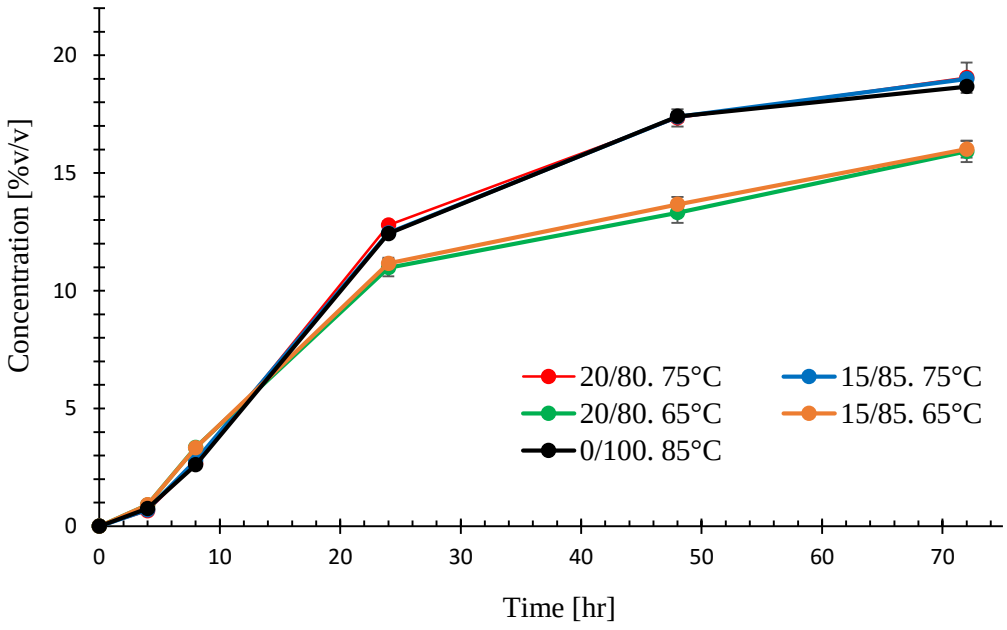


**Figure 3.1.** Ethanol profiles during fermentation of samples liquefied at different temperatures (means of three observations). Bars are ±1 standard deviation of the mean.

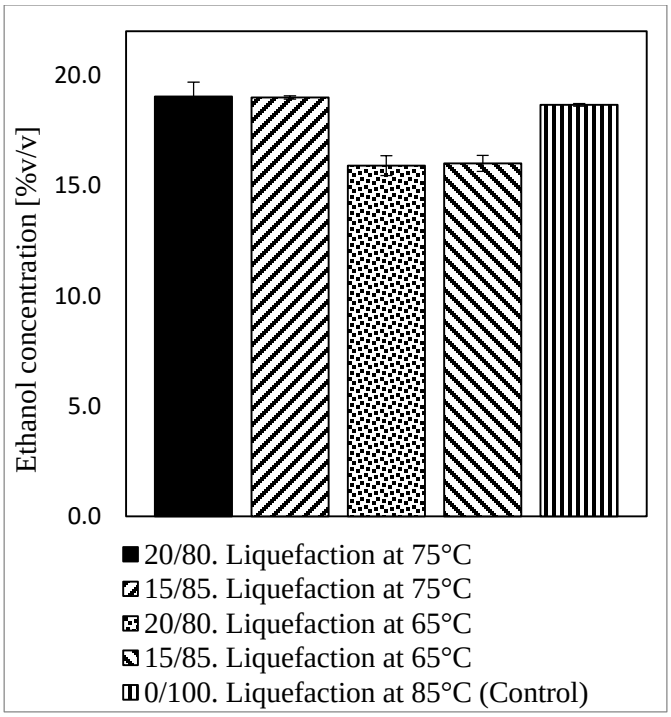


**Figure 3.2.** Final ethanol concentrations after fermentation of samples liquefied at different temperatures (means of three observations). Bars are ±1 standard deviation of the mean.

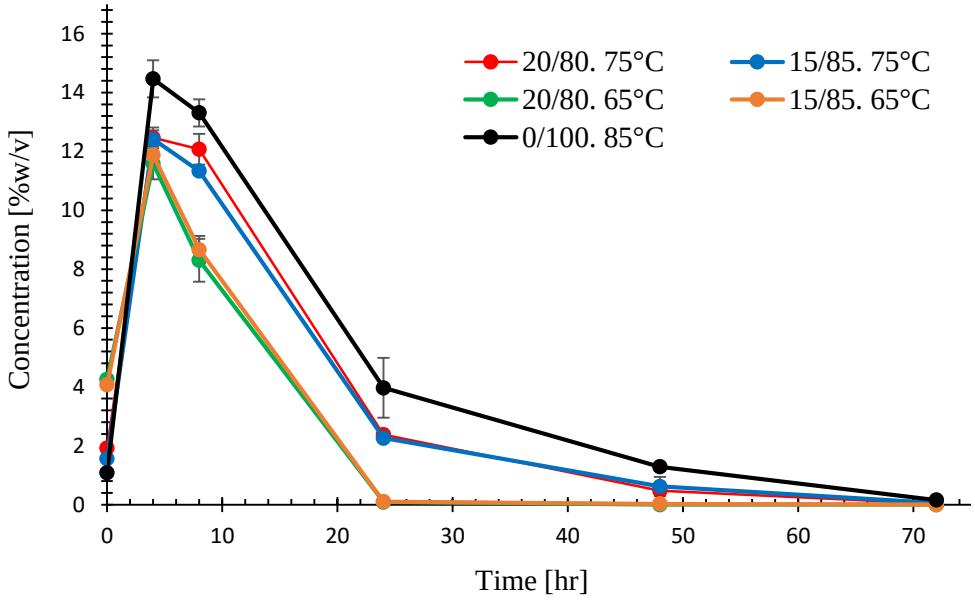


**Figure 3.3.** Glucose profiles during fermentation (means of three observations). Bars are ±1 standard deviation of the mean.

Treatments liquefied at 75˚C reached final ethanol concentrations (19.0% v/v) not different than those achieved by the control treatment (18.7% v/v) (Fig. 3.2). Samples liquefied at 65˚C resulted in lower ethanol concentrations (16.0% v/v) in comparison with those liquefied at 75˚C and with the control treatment.

Glucose profiles for all treatments had peak values at the 4 hr sampling point (Fig. 3.3). Values ranged from 11.6 to 14.5% w/v, below the inhibitory level (> 15% w/v) reported by Thatipamala et al (1992). Glucose concentrations after SSF for samples liquefied at 75˚C and for the control treatment were below 0.2% w/v, indicative of total glucose consumption during fermentation. At the end of fermentation, samples liquefied at 65˚C had glucose concentrations below the HPLC's detection range (0.0% w/v for statistical purposes). For these treatments, glucose levels were negligible after 24 hr, the time point at which fermentation rates decreased.

Ethanol yields and conversion efficiencies of the control treatment and samples liquefied at 75˚C were not different (Table 3.3). Mean ethanol yield and conversion efficiency were 481 L/ton and 92.5%, respectively. Ethanol yields and conversion efficiencies for treatments liquefied at 65˚C were not different from each other. However, they were lower than those of the control treatment and the samples liquefied at 75˚C. For the treatment with liquefaction at 65˚C, mean ethanol yield was 397 L/ton and conversion efficiency was 76.5%. For treatments at 65˚C, glucose profiles, low fermentation rates after 24 hr and low final ethanol concentrations are indicative of an incomplete conversion of starch into fermentable sugars.

**Table 3.3.** Ethanol yields and conversion efficiencies (means of three observations).

| Treatment | | Parameters | |
|---|---|---|---|
| Corn Composition [% amylase / % yellow dent] | Liquefaction Temperature [˚C] | EtOH yield (db)[a,b] [L/ton] | Conversion Efficiency[a,b] [%] |
| 20/80 | 75 | 490 a | 94 a |
| 15/85 | 75 | 488 a | 94 a |
| 20/80 | 65 | 393 b | 76 b |
| 15/85 | 65 | 399 b | 77 b |
| 0/100 | 85 | 478 a | 92 a |

[a] Means followed by the same letter in the same column are not different.

[b] LSD for ethanol yield and conversion efficiency were 24 L/ton and 4%, respectively.

## 3.4. Conclusions

The inclusion of 15 or 20% amylase corn in corn mixtures employed for the dry grind production of ethanol allowed reducing the liquefaction temperature by 10˚C (to 75˚C) without affecting final ethanol concentrations, ethanol yields and conversion efficiencies. This could translate into energy savings and a reduction of production costs.

# Chapter 4. Effects of Amylase Corn on Viscosity Profiles during Liquefaction

## 4.1. Introduction

The liquefaction step of the dry grind process consists of converting starch contained in corn kernels into dextrins. In dry grind commercial plants, viscosity achieved after liquefaction has an influence on the subsequent processing of the corn slurry. High slurry viscosities can be disadvantageous for multiple reasons. One is the increase of friction losses, which causes pumping and mixing systems to consume more energy (Bakshi and Smith 1984). Additionally, heat transfer is affected negatively (Sahin 2002). Moreover, viscous mashes retain more $CO_2$ in solution (Dahod 1993) affecting the performance of *Saccharomyces cerevisiae* during fermentation (Jones and Greenfield 1982).

In the dry grind process, the operation at high solids concentration is desirable because it reduces water consumption and energy demand of distillation, evaporation and drying operations (Taylor et al 2000). However, slurry viscosity increases with solids content (Fan et al 2003).

A liquefaction process capable of achieving low viscosities, working with slurries at high solids, is needed. We evaluated the viscous behavior of corn slurries at 36% w/w, using two exogenous commercial alpha-amylases and amylase corn at 15% inclusion rate as liquefying agents.

## 4.2. Materials and Methods

### 4.2.1. Materials

Two corn varieties, yellow dent and alpha-amylase corn, were used. Both were harvested in October, 2014. In this study, two types of exogenous alpha-amylases were used for the

liquefaction of samples containing no amylase corn. These enzymes, obtained from two commercial enzyme companies, were referred to as AA-1 and AA-2. The optimal temperature for both alpha-amylases was 85°C and the ideal pH was 5.1 for AA-1 and 5.7 to 5.8 for AA-2. The solvent utilized for these experiments was deionized water, produced in our facilities. Slurry pH adjustments were performed with sulfuric acid 10*N* (Ricca Chemical Company (Arlington, TX).

### 4.2.2. Methods

#### 4.2.2.1. Moisture and Starch Content Analyses

Ground corn samples were analyzed for determining their moisture and starch contents. A conventional oven method (AACC International 2000a) was used for determining ground corn moisture content. Starch concentrations were determined following a total starch assay procedure (AACC International 2000b), utilizing an assay kit produced by Megazyme International Ireland Limited (Bray, Wicklow, Ireland). Mean starch and moisture contents were 71.9 and 10.2%, respectively.

#### 4.2.2.2. Liquefaction and Production of Viscosity Profiles

A viscometer (Rapid Visco Analyzer, RVA-4, Newport Scientific, Warriewood, Australia) was used for conducting liquefaction of corn slurries. During liquefaction, a viscosity profile was produced for each of the samples. Slurries were comprised of 25 g (db) ground corn combined with deionized water to reach a solids concentration of 36% w/w.

Corn slurry preliquefaction viscosities were measured before enzyme addition. Samples were placed in the RVA, with constant stirring at 80 rpm and a temperature of 32°C for 10 min.

This period provided time for stabilizing and recording preliquefaction viscosity values. After this measurement, slurry pH values were adjusted to the optimal level for each particular treatment. Alpha-amylases AA-1 and AA-2 were added to treatments that included no amylase corn. Each enzyme was added at the upper limit of the dosage recommended by the manufacturer (0.31 mg/g (db) corn for AA-1 and 0.24 mg/g (db) corn for AA-2).

In the first stage of liquefaction, corn slurry samples were heated rapidly from room temperature to 32°C. The temperature was increased at 18°C/min until reaching 85°C and kept for 90 min to allow for starch gelatinization and hydrolysis. To measure final viscosities, slurries were cooled to 32°C at a rate of 5°C/min and held for 10 min.

#### 4.2.2.3. Experimental Design and Data Analysis

Three treatments were evaluated: one of 15% alpha-amylase corn and 85% yellow dent corn and two of 100% yellow dent corn (Table 4.1). No enzymes were added to the treatment with 15% amylase corn, as this variety contained alpha-amylase. AA-1 and AA-2 were each assigned to one of the 100% yellow dent corn treatments.

**Table 4.1.** Treatments used to evaluate the effect of different alpha-amylase sources on final liquefaction viscosity of corn slurries at high solids (36% w/w).

| Corn Composition [% amylase / % yellow dent] | Alpha-Amylase Added |
|---|---|
| 15/85 | None |
| 0/100 | AA-1 |
| 0/100 | AA-2 |

Liquefactions were conducted with three replications. Experimental conditions, such as temperatures, stirring speed, pH and alpha-amylase dose, were constant for each treatment. RVA viscosity data were collected through computational software (TCW3, Perten Instruments, Sidney, Australia). Viscosity profiles were evaluated to determine effects of alpha-amylase sources on final liquefaction viscosity of corn slurries at high solids (36% w/w). Initial, peak, setback, final and breakdown viscosity means were analyzed statistically using SAS (SAS Institute, Cary, NC). ANOVA and LSD tests with $p<0.05$ were used to compare means among treatments.

## 4.3. Results and Discussion

Corn slurry viscosity profiles were evaluated using a 90 min liquefaction process (Fig. 4.1). During initial heating, the temperature increased from 32 to 85°C. The heating process in excess water caused starch granules to hydrate progressively and swell (Tester et al 2006). This phenomenon resulted in a higher volume fraction of granules in the medium and a corresponding increase in slurry viscosity (Bagley and Christianson 1982). Starch granules swell to a point at which their granular structure is lost and gelatinization occurs. After this occurs, alpha-amylase is able to digest starch granules. Exogenous and endogenous alpha-amylases used in these experiments approached their optimum action range as the temperature increased to 85˚C. Amylolytic effect on slurry viscosities can be observed in Fig. 4.1. Viscosities increased with temperature and peaked as the system reached 85˚C. As the process continued, the enzymes were able to break down amylose and amylopectin chains released from starch granules. This process led to a progressive viscosity reduction, reaching values from 100 to 200 cP.

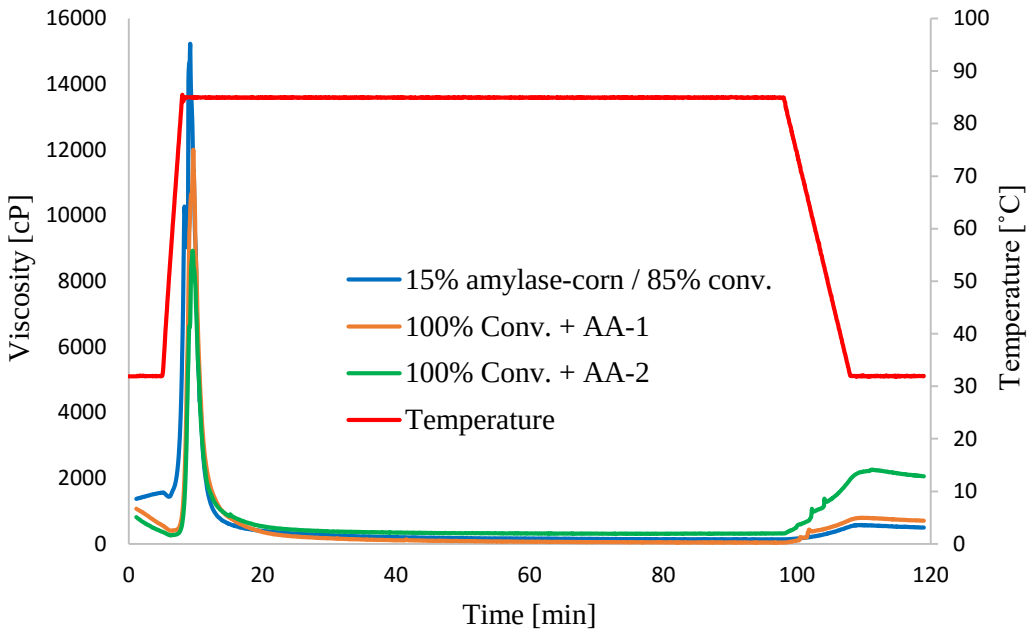


**Figure 4.1.** Liquefaction viscosity profiles obtained from Rapid Visco Analyzer, through TCW3 (means of three observations).

After completing a 90 min liquefaction period at 85˚C, system temperature was decreased to 32˚C. Upon cooling, the corn slurry samples experienced a final viscosity increase, which was caused by a phenomenon called retrogradation, in which molecular chains reorganize in an ordered structure (Sandu and Singh 2007). The treatments at 100% conventional corn (liquefied with AA-1) and at 15% amylase corn achieved similar final viscosities (Fig. 4.2). Comparatively, the treatment at 100% conventional corn, liquefied with AA-2, reached the highest viscosity at the end of the process. Therefore, starch hydrolysis was adequate in the first two cases; in the latter, polysaccharides left in solution recombined, causing an increase in the final viscosity.

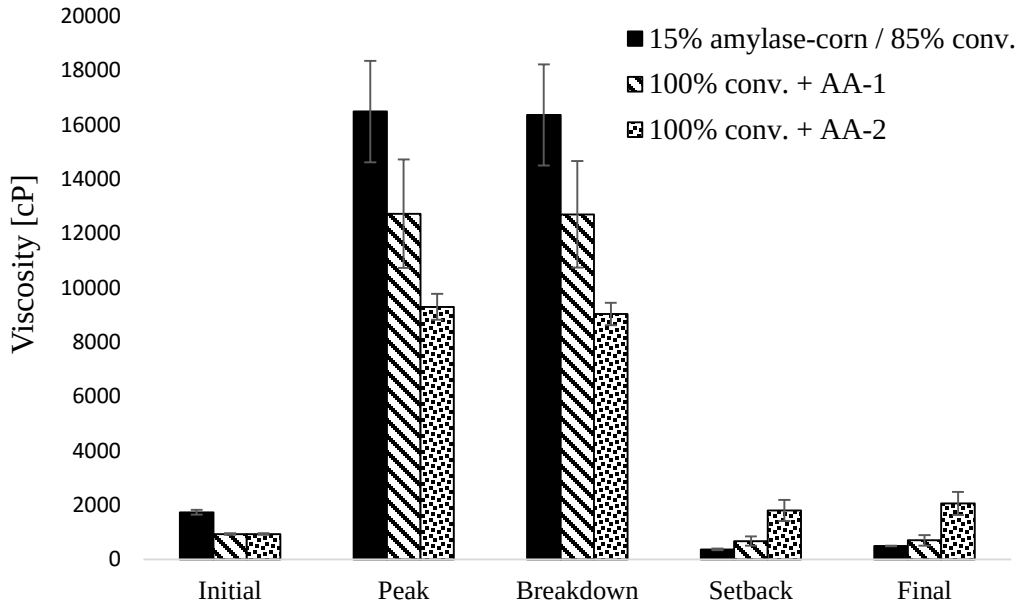


**Figure 4.2.** Initial, peak, breakdown, setback and final viscosity values observed during liquefaction (means of three observations). Bars are ±1 standard deviation of the mean.

Initial, peak, breakdown, setback and final viscosities observed in the three treatments are depicted in Fig. 4.2. The corn mixture with 15% amylase corn had higher initial viscosity than that of only yellow dent corn. The AA-2 treatment had the lowest viscosity peak, followed by AA-1 treatment. Maximum peak viscosity was reached by the treatment with 15% amylase corn. Singh et al (2006a) also observed this peak. They suggested that during the initial phase of the liquefaction process, the endogenous alpha-amylase contained in amylase corn was not entirely available. Eventually, the enzyme is released and activated becoming able to depolymerize amylose and amylopectin chains and reducing slurry viscosity. In control treatments, alpha-amylase was readily available in the slurry, as it was added externally in liquid form.

Treatments with 15% amylase corn had the highest breakdown viscosity (16400 cP). Despite developing the maximum peak among all treatments, the large breakdown capability of

amylase corn resulted in similar viscosities to those reached by AA-1 and AA-2 treatments during liquefaction.

**Table 4.2.** Viscosities observed during liquefaction of samples with 36% w/w solids at 85˚C (means of three observations).

| Corn Composition [% amylase / % yellow dent] | Alpha-Amylase Added | Viscosity [cP] | | | | |
|---|---|---|---|---|---|---|
| | | Initial[a,b] | Peak[a] | Breakdown[a] | Setback[a] | Final[a] |
| 15/85 | - | 1700 a | 16500 a | 16400 a | 400 a | 500 a |
| 0/100 | AA-1 | 900 b | 12700 b | 12700 b | 700 a | 700 a |
| 0/100 | AA-2 | 900 b | 9300 c | 9000 c | 1800 b | 2000 b |
| | **LSD** | 100 | 3200 | 3200 | 500 | 500 |

[a] Means followed by the same letter in the same column are not different.

[b] Initial viscosity values were recorded before the addition of enzymes.

Setback viscosities were not different for AA-1 and 15% amylase corn treatments. However, AA-2 treatment had the largest setback viscosity, resulting in the highest final value. Final viscosities of 15% amylase corn and AA-1 treatments were not different.

## 4.4. Conclusions

The liquefaction of corn slurries at 36% w/w solids with amylase corn inclusion level of 15% resulted in peak viscosities of 16500 cP, higher than treatments with conventional alpha-amylases. High peak viscosities observed in amylase corn treatments may limit the applicability of this technology at commercial scale.

Samples with 15% amylase corn had final liquefaction viscosities of 500 cP. Inclusion of 15% amylase corn is a viable alternative as a source of alpha-amylase and as a viscosity reducing agent. Application of this technology resulted in similar or lower final viscosities than with exogenous alpha-amylases. The high viscosity reducing capacity of amylase corn may allow processing slurries at higher solids concentration and reduce energy and water consumption.

# Chapter 5. Performance of Amylase Corn in Combination with Various Glucoamylases

## 5.1. Introduction

Economic viability of dry grind plants depends on the value obtained from the production of ethanol and coproducts such as dry distillers grains with solubles (DDGS), corn oil and $CO_2$ (Bothast and Schlicher 2005). The volume of ethanol obtained per bushel of corn has a direct impact on the dry grind facility's profitability.

One factor affecting final ethanol yields in corn dry grind plants is corn quality, which is influenced by genetics, planting environment and postharvest practices (Singh 2012). Singh and Graeber (2005) observed a 23% final ethanol concentration variability when evaluating eight planting locations and 18 hybrid types. Postharvest corn drying temperatures have an influence on ethanol yields, therefore, it is recommended to employ as low a temperature as possible during the drying process (Singh 2016). Ramchandran et al (2015) evaluated ethanol yield as a function of a 12 mo storage period. The optimum was week 12. They also observed the application of effective enzyme treatments increased final ethanol concentrations, enhancing process profitability.

Amylase corn produces and stores alpha-amylase in the seed endosperm. The inclusion of this strain in the corn feedstock can eliminate the need of exogenous alpha-amylase for starch hydrolysis (Singh et al 2006a). It can allow working with higher solids contents, reducing water consumption and the demand for natural gas and electricity. Furthermore, the inclusion of amylase corn may result in increased ethanol yields (Urbanchuk and Kowalski 2009).

We evaluated the performance of amylase corn in combination with different commercial glucoamylases in the dry grind process. Our primary goals were:

- To detect which of the treatments resulted in highest final ethanol concentration, working at 32% w/w solids.

- To determine if the inclusion of amylase corn, combined with any of the glucoamylases tested, allowed working efficiently at 36% w/w solids loading.

## 5.2. Materials and Methods

### 5.2.1. Materials

Yellow dent and amylase corn was provided by a commercial seed company. Corn was harvested in October, 2014. Two alpha-amylases and five glucoamylases were obtained from three commercial enzyme companies. Optimum pH and recommended doses are listed in Table 5.1.

Slurry pH values were adjusted with sulfuric acid 10*N* (Ricca Chemical Company, Arlington, TX). Fermentations were conducted using active dry yeast (Ethanol Red, Fermentis-Lessaffre Yeast Corporation, Milwaukee, WI) and Ricca Chemical Company (Arlington, TX) provided urea as a source of nitrogen for yeast nutrition. The solvent utilized for preparing corn slurry samples was deionized water.

**Table 5.1.** Optimum pH and recommended enzyme doses used in this study.

| Enzyme | Optimum pH | Recommended Dose[a] [mg/g (db) corn] |
|---|---|---|
| AA-1 | 5.1 | 0.31 |
| AA-2 | 5.6 to 6 | 0.24 |
| GA-1 | 3.5 to 4.5 | 0.66 |
| GA-2 | 4.5 to 5 | 0.65 |
| GA-3 | 4 to 5.5 | 0.80 |
| GA-4 | 4.5 | 0.65 |
| GA-5 | 4.5 | 0.59 |

[a] Upper level of dose recommended by the enzyme manufacturer.

### 5.2.2. Methods

#### 5.2.2.1. Moisture and Starch Content Analyses

Ground corn sample moisture content was determined following a conventional oven method (Method 44-19.01, AACC International 2000a). Starch concentrations were calculated following a total starch assay procedure (Method 76-13.01, AACC International 2000b). This procedure was conducted using an assay kit provided by Megazyme International Ireland Limited (Bray, Wicklow, Ireland). Mean moisture and starch contents were 10.2 and 71.9%, respectively.

#### 5.2.2.2. Dry Grind Procedure

Corn samples were cleaned for removing broken kernels and foreign material using a 12/64" (4.8 mm) round hole sieve. Clean corn was processed in an electric hammer mill (Retsch SK100, Glen Mills, Clifton, NJ) with rotational speed of 3,420 rpm and a screen size of 0.5 mm.

Ground samples were stored at 4˚C in sealed plastic bags. Moisture and starch content analyses were performed according to the procedures described in section 5.2.2.1.

Samples for the dry grind procedure were prepared in 500 ml stainless steel reactors. Ground corn weighing 100 g (db) was mixed with deionized water to reach the desired solids content. As a step before liquefaction, pH was adjusted to the required value (5.1±0.05 or 5.7±0.05, according to the alpha-amylase used) with sulfuric acid 10*N*. AA-1 and AA-2 were added to the control treatments, which included no amylase corn. Both enzymes were added at the upper level of the dose recommended by the manufacturer (0.31 mg/g (db) corn for AA-1 and 0.24 mg/g (db) corn for AA-2). Samples were liquefied in a programmable incubator (Labomat BFA-12, Werner Mathis AG, Switzerland) with continuous agitation. The liquefaction process was conducted at 85˚C for 150 min, as recommended by the amylase corn provider. After completing liquefaction, samples were cooled to 32˚C.

As preparation for SSF, pH of liquefied samples was adjusted to 4.50±0.05. Glucoamylases were added to each reactor according to the treatment selected. Each enzyme was applied at the upper level of the range recommended by the manufacturer (see Table 5.1). A 50% w/v urea solution was added at 2.25 mg urea/g (db) corn, as a nitrogen source for yeast nutrition. A yeast inoculate at 20% w/v was prepared and brought to logarithmic growing phase by holding it at 32˚C for 20 min in an automatic incubator (New Brunswick Innova 42R Inc/Ref Shaker, Eppendorf, Enfield, CT). Two ml of yeast inoculate was added to each fermentation reactor. SSF was carried out at 32˚C for 72 hr with constant agitation speed of 120 rpm.

#### 5.2.2.3. Metabolite Detection

The evolution of metabolite profiles during SSF was tracked by extracting 2 ml samples after 0, 4, 8, 24, 32, 48 and 72 hr. Coarse separation of the solid and liquid phases was achieved by centrifugation (Centrifuge 5415D1, Eppendorf, Germany) for five min at 15,000 *xg*. The liquid phase was cleared of solids by forcing it through a 0.2 µm syringe filter (Pall Gelman, Milford, MA) into 1 ml vials.

SSF metabolites were analyzed by high performance liquid chromatography (HPLC). The filtered liquid was injected into an ion exclusion column (Aminex HPX-87H, Bio-Rad, Hercules, CA) maintained at 50°C. Alcohols (ethanol, methanol and glycerol), sugars (DP4+, DP3, DP2, fructose and glucose) and organic acids (lactic, succinic and acetic) were quantified by a refractive index detector at 35°C (model 2414, Waters Corporation, Milford, MA).

#### 5.2.2.4. Experimental Design and Data Analysis

We evaluated final ethanol concentrations reached by fermentation, including 15% amylase corn in the feedstock and combining it with different glucoamylases. Two corn mixtures were tested: one comprised of 15% amylase and 85% yellow dent corn and a second one containing 100% yellow dent corn. Seven treatments (Table 5.2) were evaluated using three replicates. Treatments C1 and C2 were used as controls. The same treatments were utilized for two independent experiments: one at conventional solids content (32% w/w) and the other at high solids content (36% w/w).

Liquefaction and fermentation conditions, such as pH, temperature, doses of urea, glucoamylase and yeast, were constant for each treatment. Samples were analyzed in duplicate using HPLC.

**Table 5.2.** Treatments tested for evaluating final ethanol concentrations. The same design was used for performing the experiment at 32 and 36% w/w solids content.

| Treatment | Corn Comp. [% amylase / % yellow dent] | Alpha-Amylase | Glucoamylase |
|---|---|---|---|
| T1 | 15/85 | - | GA-1 |
| T2 | 15/85 | - | GA-2 |
| T3 | 15/85 | - | GA-3 |
| T4 | 15/85 | - | GA-4 |
| T5 | 15/85 | - | GA-5 |
| C1 | 0/100 | AA-1 | GA-1 |
| C2 | 0/100 | AA-2 | GA-3 |

Statistical analyses were conducted using SAS (SAS Institute, Cary, NC). ANOVA and LSD tests with $p<0.05$ were conducted for comparing means among treatments.

#### 5.2.2.5. Additional Experiment: Reduction of Liquefaction Time

The experiments described above resulted in unusual residual glucose concentrations. Due to this finding, additional dry grind experiments were performed for evaluating SSF glucose profiles and final glucose concentrations in a modified process. This additional procedure was conducted using two treatments with 32% w/w solids from the initial study. Treatments were selected randomly. Treatments tested were T2, which included 15% amylase corn, and C1, comprised of 100% yellow dent corn. The procedure was conducted under conditions reported in section 5.2.2.2, except liquefaction time, which was reduced from 150 to 90 min.

## 5.3. Results and Discussion

Variable final ethanol concentrations were detected (Table 5.3). Working at 32% w/w solids, treatments T2 and T3 achieved highest final ethanol concentrations of 19% v/v. These values were not different from those reached by control treatments C1 and C2. Final ethanol levels of treatments T1, T4 and T5 were not different from each other or C2 but were lower than in treatments T2, T3 and C1. Treatments T3 and C2, both treated with GA-3, had similar ethanol production. Treatment T1 resulted in lower ethanol concentration in comparison with C1, despite being processed with the same glucoamylase, GA-1.

**Table 5.3.** Fermentation parameters (means of three observations).

| | 32% w/w | | 36% w/w | |
|---|---|---|---|---|
| Treatment | Final EtOH Conc.[a,b] [%v/v] | Residual Glucose [%w/v] | Final EtOH Conc.[a,c] [%v/v] | Residual Glucose [%w/v] |
| T1 | 17.9 b | 1.9 | 19.0 ab | 4.1 |
| T2 | 19.0 a | 1.3 | 18.9 ab | 4.1 |
| T3 | 19.0 a | 0.7 | 19.1 ab | 4.4 |
| T4 | 17.7 b | 1.5 | 18.7 b | 3.7 |
| T5 | 17.1 b | 1.3 | 18.7 b | 3.6 |
| C1 | 18.9 a | 0.7 | 19.2 a | 3.2 |
| C2 | 18.2 ab | 1.2 | 19.2 a | 3.1 |

[a] Means followed by the same letter in the same column are not different.

[b] LSD for final EtOH concentration was 0.8% v/v.

[c] LSD for final EtOH concentration was 0.5% v/v.

The experiment at 36% w/w solids resulted in maximum ethanol concentrations ranging from 18.9 to 19.2% v/v for treatments T1, T2, T3, C1 and C2. These values were not different

from each other. Treatments T4 and T5 had the lowest final ethanol levels, but were not different from those reached by T1, T2 or T3.

At 36% w/w solids, glucose levels ranged from 3.1 to 4.4% w/v. Therefore, none of the treatments tested at this solids level achieved complete sugar consumption. Incomplete utilization of glucose may have resulted from yeast inhibition at ethanol levels of approximately 19% v/v. The experiment at 32% w/w solids, which is a solids content typically used in dry grind plants (Taylor et al 2010), resulted in final glucose concentrations between 0.7 and 1.9% w/v. These results were unexpected, as fermentation of slurries at conventional solids content commonly reaches a total consumption of glucose.

### 5.3.1 Effect of Reduced Liquefaction Time on Residual Saccharides

Fermentation of samples under treatment T2, liquefied for 90 min, resulted in a low concentration of residual saccharides, especially that of glucose. The magnitude of residual saccharide reduction achieved by the modified procedure is shown in Fig. 5.1. In treatment T2, only DP4+ had a larger final concentration (56% increase). However, levels of DP3, DP2 and glucose were lower. DP3 and DP2 decreased by 56 and 42%, respectively. Residual glucose was 0.1% w/v, 92% lower than that observed in the original procedure. In the case of treatment C1 (Fig. 5.2), the final concentration of DP4+ increased by 14% while DP2 and fructose concentrations decreased by 27 and 49%, respectively. Residual glucose was 0.03% w/v, 96% lower than the original procedure. Despite resulting in lower concentration of residual saccharides, SSF of samples liquefied for 90 min did not yield higher final ethanol levels than samples liquefied for 150 min.

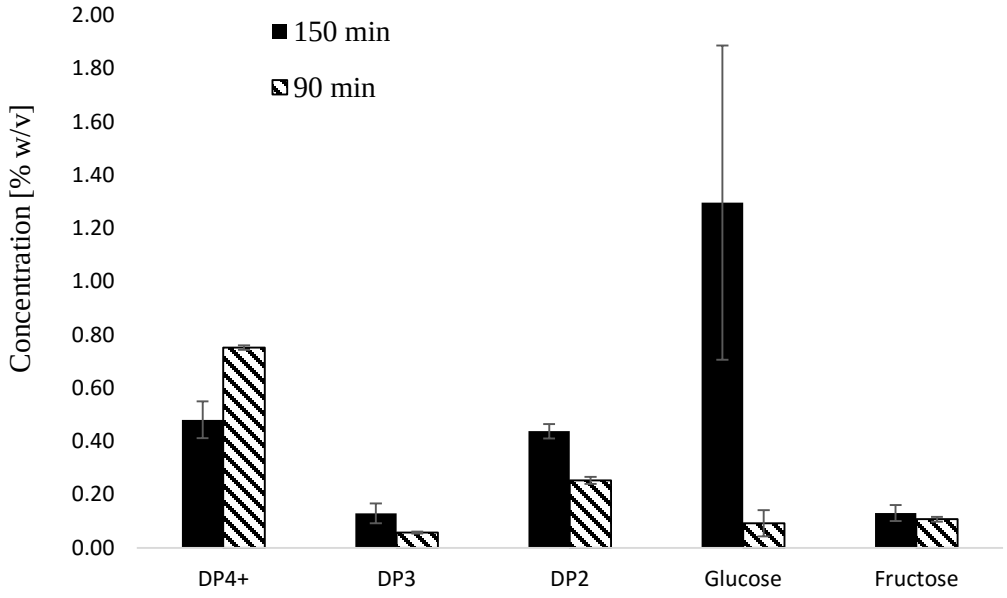


**Figure 5.1.** Residual saccharides comparison in fermentation of samples liquefied for 90 and 150 min (means of three observations). Bars are ±1 standard deviation of the mean. Treatment: T2.

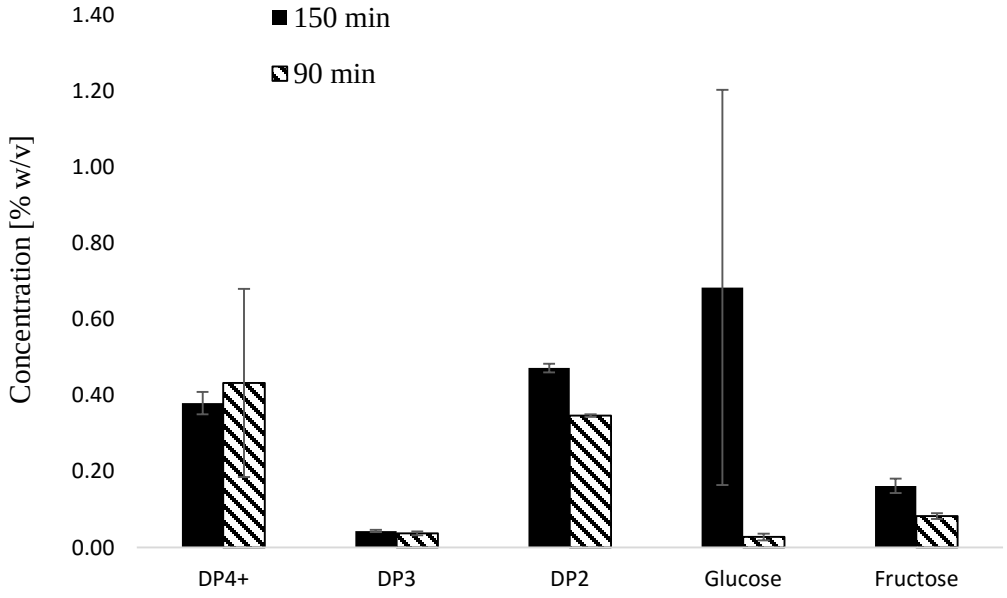


**Figure 5.2.** Residual saccharides comparison in fermentation of samples liquefied for 90 and 150 min (means of three observations). Bars are ±1 standard deviation of the mean. Treatment: C1.

Glucose profiles developed during SSF of treatments T2 and C1 are depicted in Fig. 5.3 and 5.4, respectively. In both cases, SSF of samples liquefied for 90 min resulted in high glucose production rates and negligible glucose concentrations at 72 hr. Glucose production in samples liquefied for 150 min proceeded more slowly; glucose was present after finishing SSF.

In treatments T2 and C1, lower glucoamylase rates observed in samples liquefied for 150 min were probably due to a large concentration of short chain saccharides in solution. Liquefaction of samples for 150 min provided enough time for alpha-amylase to break down amylose and amylopectin chains into oligosaccharides, on which glucoamylase acts slowly. In such conditions, glucoamylase produced fermentable sugars at late stages of fermentation, when the stress exerted over yeast by ethanol was high. This stress led to an incomplete consumption of fermentable sugars by yeast.

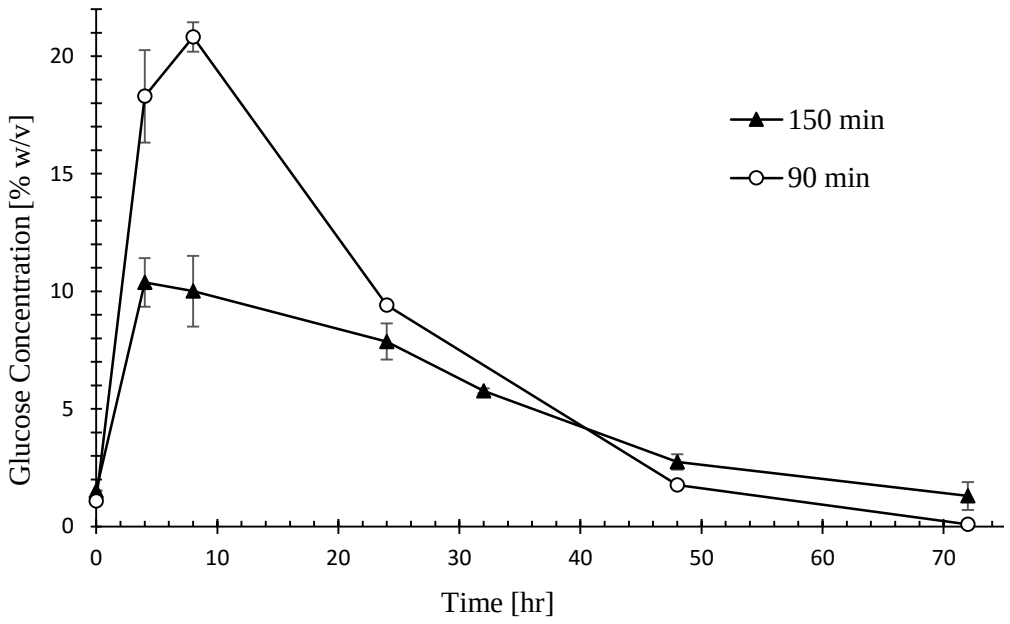


**Figure 5.3.** Glucose profiles during fermentation of samples liquefied for 90 and 150 min (means of three observations). Bars are ±1 standard deviation of the mean. Treatment: T2.

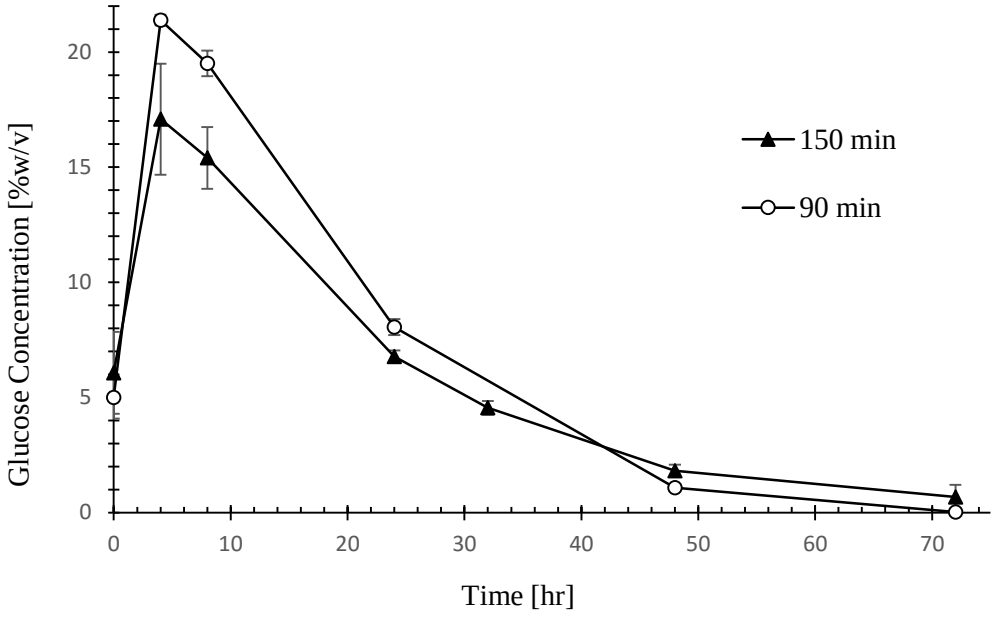


**Figure 5.4.** Glucose profiles during fermentation of samples liquefied for 90 and 150 min (means of three observations). Bars are ±1 standard deviation of the mean. Treatment: C1.

Glucose peaks achieved by T2 and C1 during 150 min liquefaction were 10.4 and 17.1% w/v, respectively. This difference was indicative of a lower glucose production rate in treatment T2. With 150 min liquefaction, the inclusion of amylase corn at 15% resulted in an additional detrimental factor for glucoamylase performance. This factor could be high concentration of α-1-6 linked saccharides after liquefaction.

## 5.4. Conclusions

Inclusion of amylase corn at 15% in the dry grind process, in combination with certain glucoamylases, yielded final ethanol concentrations comparable to those achieved with 100% yellow dent corn. However, the fermentation of samples at 36% w/w solids was not efficient, as high levels of residual glucose remained after SSF.

Fermentation of corn slurry samples liquefied for 150 min resulted in residual glucose at the end of SSF, regardless of corn composition and solids content. Working with a solids content of 32% w/w, reduction of liquefaction time to 90 min resulted in lower amounts of residual saccharides and negligible glucose concentrations upon completion of SSF.

## References


AACC International. 2000a. Approved methods of the American Associacion of Cereal Chemists. 10th ed. Method 44-19.01. AACC International: St. Paul, MN.

AACC International. 2000b. Approved methods of the American Associacion of Cereal Chemists. 11th ed. Method 76-13.01. AACC International: St. Paul, MN.

Aiyer, P. 2005. Amylases and their applications. Afr. J. Biotechnol. 4:1525-1529.

Bagley, E., and Christianson, D. 1982. Swelling capacity of starch and its relationship to suspension viscosity - effect of cooking time, temperature and concentration. J. Texture Stud. 13:115-126.

Bakshi, A., and Smith, D. 1984. Effect of fat content and temperature on viscosity in relation to pumping requirements of fluid milk products. J. Dairy Sci. 67:1157-1160.

Bothast, R., and Schlicher, M. 2005. Biotechnological processes for conversion of corn into ethanol. Appl. Microbiol. Biotechnol. 67:19-25.

Brown, S., Oliver, S., Harrison, D., and Righelato, R. 1981. Ethanol inhibition of yeast growth and fermentation: Differences in the magnitude and complexity of the effect. Eur. J. Appl. Microbiol. 11:151-155.

Casey, G., and Ingledew, W. 1986. Ethanol tolerance in yeasts. CRC Cr. Rev. Microbiol. 13:219-280.

Crabb, W., and Mitchinson, C. 1997. Enzymes involved in the processing of starch to sugars. Trends Biotechnol. 15:349-352.

Dahod, S. 1993. Dissolved carbon dioxide measurement and its correlation with operating parameters in fermentation processes. Biotechnol. Progr. 9:655-660.

Daugulis, A., Axford, D., and McLellan, P. 1991. The economics of ethanol production by extractive fermentation. Can. J. Chem. Eng. 69:488-497.

Devantier, R., Pedersen, S., and Olsson, L. 2005. Characterization of very high gravity ethanol fermentation of corn mash. Effect of glucoamylase dosage, pre-saccharification and yeast strain. Appl. Microbiol. Biotechnol. 68:622-629.

Dintzis, F., and Bagley, E. 1995. Effects of thermomechanical processing on viscosity behavior of corn starches. J. Rheol. 39:1483-1495.

Dokic, L., Jakovljevic, J., and Dokic, P. 2004. Relation between viscous characteristics and dextrose equivalent of maltodextrins. Starch-Stärke 56:520-525.

Fan, Z. F., South, C., Lyford, K., Munsie, J., van Walsum, P., and Lynd, L. 2003. Conversion of paper sludge to ethanol in a semicontinuous solids-fed reactor. Bioproc. Biosyst. Eng. 26:93-101.

Hii, S., Tan, J., Ling, T., and Bin Ariff, A. 2012. Pullulanase: role in starch hydrolysis and potential industrial applications. Enzyme Res. 2012: Article ID 921362, 14 pages.

Huang, H., Qureshi, N., Chen, M., Liu, W., and Singh, V. 2015. Ethanol production from food waste at high solids content with vacuum recovery technology. J. Agr. Food Chem. 63:2760-2766.

Jones, A., and Ingledew, W. 1994a. Fuel alcohol production: appraisal of nitrogenous yeast foods for very high gravity wheat mash fermentation. Process Biochem. 29:483-488.

Jones, A., and Ingledew, W. 1994b. Fuel alcohol production: assessment of selected commercial proteases for very high gravity wheat mash fermentation. Enzyme Microbiol. Technol. 16:683-687.

Jones, R., and Greenfield, P. 1982. Effect of carbon dioxide on yeast growth and fermentation. Enzyme Microbiol. Technol. 4:210-223.

Kaur, P. 2010. Enzymatic and process technologies to increase corn dry-grind slurry solids. MS Thesis. Urbana, IL: University of Illinois at Urbana-Champaign, Department of Agricultural and Biological Engineering.

Kaur, P., Rausch, K., Tumbleson, M., and Singh, V. 2011. Enzymatic process for corn dry-grind high-solids fermentation. Cereal Chem. 88:429-433.

Kondo, A., Shigechi, H., Abe, M., Uyama, K., Matsumoto, T., Takahashi, S., Ueda, M., Tanaka, A., Kishimoto, M., and Fukuda, H. 2002. High-level ethanol production from starch by a flocculent Saccharomyces cerevisiae strain displaying cell-surface glucoamylase. Appl. Microbiol. Biotechnol. 58:291-296.

Kwiatkowski, J., McAloon, A., Taylor, F., and Johnston, D. 2005. Modeling the process and costs of fuel ethanol production. Ind. Crop. Prod. 23:288-296.

Marin-Navarro, J., and Polaina, J. 2011. Glucoamylases: structural and biotechnological aspects. Appl. Microbiol. Biotechnol. 89:1267-1273.

Mohagheghi, A., Tucker, M., Grohmann, K., and Wyman, C. 1992. High solids simultaneous saccharification and fermentation of pretreated wheat straw to ethanol. Appl. Biochem. Biotechnol. 33:67-81.

Morris, V. 1990. Starch gelation and retrogradation. Trends Food. Sci. Technol. 1:2-6.

Naidu, K., Singh, V., Johnston, D., Rausch, K., and Tumbleson, M. 2007. Effects of ground corn particle size on ethanol yield and thin stillage soluble solids. Cereal Chem. 84:6-9.

Nigam, P., and Singh, D. 1995. Enzyme and microbial systems involved in starch processing. Enzyme Microbiol. Technol. 17:770-778.

Norman, B. 1982. A novel debranching enzyme for application in the glucose syrup industry. Starch-Stärke 34:346-351.

Norouzian, D., Akbarzadeh, A., Scharer, J., and Moo Young, M. 2006. Fungal glucoamylases. Biotechnol. Adv. 24:80-85.

Power, R. 2003. Enzymatic conversion of starch to fermentable sugars. Pages 34-43 in: The Alcohol Textbook. A Reference for the Beverage, Fuel and Industrial Alcohol Industries, 4th Edition. Jacques, K., Lyons, T., and Kelsall, D., eds. Nottingham University Press: Nottingham, UK.

Ramchandran, D., Johnston, D., Tumbleson, M., Rausch, K., and Singh, V. 2015. Seasonal variability in ethanol concentrations from a dry grind fermentation operation associated with incoming corn variability. Ind. Crop. Prod. 67:155-160.

RFA. 2015a. Pocket guide to ethanol. Retrieved from Renewable Fuels Association Database. Washington D.C. Available at www.ethanolrfa.org. Accesed 2 Oct. 2015.

RFA. 2015b. Fueling a nation. Feeding the world. The role of the U.S. ethanol industry in food and feed production. Retrieved from Renewable Fuels Association Database. Washington D.C. Available at www.ethanolrfa.org. Accesed 2 Oct. 2015.

Robertson, R., Wong, W., Lee, C., Wagschal, K., Smith, M., and Orts, W. 2006. Native or raw starch digestion. A Key step in energy efficient biorefining of grain. J. Agr. Food Chem. 54:353-365.

Russell, I. 2003. Understanding yeast fundamentals. Pages 85-119 in: The Alcohol Textbook. A Reference for the Beverage, Fuel and Industrial Alcohol Industries, 4th Edition. Jacques, K., Lyons, T., and Kelsall, D., eds. Nottingham University Press: Nottingham, UK.

Saha, B., and Zeikus, J. 1989. Microbial glucoamylases: biochemical and biotechonological features. Starch-Stärke 41:57-64.

Sahin, A. 2002. Entropy generation and pumping power in a turbulent fluid flow through a smooth pipe subjected to constant heat flux. Int. J. Exergy 2:314-321.

Sandhu, K., and Singh, N. 2007. Some properties of corn starches II: Physicochemical, gelatinization, retrogradation, pasting and gel textural properties. Food Chem. 101:1499-1507.

Sharma, V., Rausch, K., Tumbleson, M., and Singh, V. 2007. Comparison between granular starch hydrolyzing enzyme and conventional enzymes for ethanol production from maize starch with different amylose: amylopectin ratios. Starch-Stärke 59:549-556.

Shihadeh, J., Huang, H., Rausch, K., Tumbleson, M., and Singh, V. 2014. Vacuum stripping of ethanol during high solids fermentation of corn. Appl. Biochem. Biotechnol. 173:486-500.

Singh, V. 2012. Effect of corn quality on bioethanol production. Biocatal. Agric. Biotechnol. 1:353-355.

Singh, V. 2016. Corn quality, cleaning and grinding. New Technologies in Ethanol Production, short course offered by the Department of Agricultural and Biological Engineering. University of Illinois at Urbana-Champaign.

Singh, V., and Graeber, J. 2005. Effect of corn variability and planting location on dry grind ethanol production. Trans. ASAE 48:709-714.

Singh, V., Batie, C., Aux, G., Rausch, K., and Miller, C. 2006a. Dry-grind processing of corn with endogenous liquefaction enzymes. Cereal Chem. 83:317-320.

Singh, V., Batie, C., Rausch, K., and Miller, C. 2006b. Wet-milling and dry-milling properties of dent corn with addition of amylase corn. Cereal Chem. 83:321-323.

Singh, V., Johnston, D., Naidu, K., Rausch, K., Belyea, R., and Tumbleson, M. 2004. Comparison of modified dry-grind corn processes for fermentation characteristics and DDGS composition. Cereal Chem. 82:187-190.

Solomon, B., Barnes, J., and Halvorsen, K. 2007. Grain and cellulosic ethanol: History, economics, and energy policy. Biomass Bioenerg. 31:416-425.

Srinivasan, R., Moreau, R., Rausch, K., and Belyea, R. 2005. Separation of fiber from distillers dried grains with solubles (DDGS) using sieving and elutriation. Cereal Chem. 82:528-533.

Stominska, L., and Maczynski, P. 1985. Studies on the application of pullulanase in starch saccharification process. Starch-Stärke 37:386-390.

Taylor, F., Kurantz, M., Goldberg, N., McAloon, A., and Craig, J. 2000. Dry-grind process for fuel ethanol by continuous fermentation and stripping. Biotechnol. Progr. 16:541-547.

Taylor, F., Marquez, M., Johnston, D., Goldberg, N., and Hicks, K. 2010. Continuous high-solids corn liquefaction and fermentation with stripping of ethanol. Bioresource Technol. 101:4403-4408.

Tester, R., Qi, X., and Karkalas, J. 2006. Hydrolysis of native starches with amylases. Anim. Feed Sci. Technol. 130:39-54.

Thatipamala, R., Rohani, S., and Hill, G. 1992. Effects of high product and substrate inhibitions on the kinetics and biomass product yields during ethanol batch fermentation. Biotechnol. Bioeng. 40:289-297.

Thomas, K., Hynes, H., and Ingledew, W. 1994. Effects of particulate materials and osmoprotectants on very-high-gravity ethanolic fermentation by Saccharomyces cerevisiae. Appl. Environ. Microbiol. 60:1519-1524.

Thomas, K., Hynes, S., and Ingledew, W. 1996. Practical and theoretical considerations in the production of high concentrations of alcohol by fermentation. Process Biochem. 31:321-331.

Thomas, K., Hynes, S., Jones, A., and Ingledew, W. 1993. Production of fuel alcohol from wheat by VHG technology. Appl. Biochem. Biotechnol. 43:211-226.

U.S. Grains Council. 2012. Chapter 2: Ethanol production and its co-products - dry-grind and wet milling processes. Pages 16-26 in: A Guide to Distiller's Dried Grains with Solubles (DDGS), 3rd edition. Edited by U.S. Grains Council.

Urbanchuk, J., and Kowalski, D. 2009. Corn amylase: improving the efficiency and environmental footprint of corn to ethanol through plant biotechnology. AgBioForum 12:149-154.

Uthumporn, U., Shariffa, Y., and Karim, A. 2012. Hydrolysis of native and heat-treated starches at sub-gelatinization temperature using granular starch hydrolyzing enzyme. Appl. Biochem. Biotechnol. 166:1167-1182.

van der Maarel, M., van der Veen, B., Uitdehaag, J., Leemhuis, H., and Dijkhuizen, L. 2002. Properties and applications of starch-converting enzymes of the alpha-amylase family. J. Biotechnol. 94:137-155.

Wang, P., Singh, V., Xue, H., Johnston, D., Rausch, K., and Tumbleson, M. 2007. Comparison of raw starch hydrolyzing enzyme with conventional liquefaction and saccharification enzymes in dry-grind corn processing. Cereal Chem. 84:10-14.

Wang, Y., and Wang, L. 2000. Structures and properties of commercial maltodextrins from corn, potato, and rice starches. Starch-Stärke 52:296-304.

Weemaes, C., De Cordt, S., Goossens, K., Ludikhuyze, L., Hendrickx, M., Heremans, K., and Tobback, P. 1995. High pressure, thermal, and combined pressure-temperature stabilities of alpha-amylases from bacillus species. Biotechnol. Bioeng. 50:49-56.

Zhao, W., Nie, Y., Mu, X., Zhang, R., and Xu, Y. 2015. Enhancement of glucose production from maltodextrin hydrolysis by optimisation of saccharification process using mixed enzymes involving novel pullulanase. Int. J. Food Sci. Technol. 50:2672-2681.